

1966

Cellular and physiological changes during secretion in crayfish hepatopancreas

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**LOIZZI, Robert Francis, 1935-
CELLULAR AND PHYSIOLOGICAL CHANGES
DURING SECRETION IN CRAYFISH HEPATO-
PANCREAS.**

**Iowa State University of Science and Technology
Ph.D., 1966
Zoology**

University Microfilms, Inc., Ann Arbor, Michigan

CELLULAR AND PHYSIOLOGICAL CHANGES DURING
SECRETION IN CRAYFISH HEPATOPANCREAS

by

Robert Francis Loizzi

A Dissertation Submitted to the
Graduate Faculty in Partial Fulfillment of
The Requirements for the Degree of
DOCTOR OF PHILOSOPHY

Major Subject: Cell Biology

Approved:

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1966

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INTRODUCTION

The hepatopancreas as a research tool

Twentieth century biology is typified by a willingness to assimilate concepts and techniques from other natural sciences in order to synthesize an integrated understanding of life processes. If the multidisciplined effort is the currently favored approach toward biological problems, then the crustacean hepatopancreas is, simultaneously, one of the more useful and neglected organs in contemporary research.

First, let us consider the following hypothetical system: a monolayer of epithelial cells on a basement membrane in the form of a long, very slender triangle. The monolayer is more or less divided into regions such that mitotic divisions are restricted to the apex of the triangle and large secretory cells are located in a broad zone which takes up approximately 50% of the triangle between apex and base. In addition to undifferentiated cells, at least three specialized cell types (absorptive, synthetic, and secretory) are fairly well localized in the various regions. The positioning of the cells, however, does not imply a static condition in the monolayer; there is continued migration and differentiation of cells both from the region of mitosis to the other zones and also within each zone. Thus, the sys-

tem would be useful in studying cell secretion, differentiation and migration, with the added advantage that cell types are easily located in their respective regions.

In addition to secretion, the monolayer carries out a wide variety of interesting functions at the cellular level. The region adjacent to the apex of the triangle is specialized for absorption of foodstuffs and the cells in this region resemble those found in the vertebrate small intestine. In fact, the monolayer does carry on nearly all of the absorptive functions normally associated with the gut. The monolayer is highly specialized for nutrient storage and specific cells respond to hormonal influences by either retaining or releasing massive amounts of glycogen and lipids and by controlling the level of blood sugar. Besides metabolite storage, curious, metal-containing granules are located in specific cells. Granules containing salts of copper, iron or calcium may also be stored in, or mobilized from, the monolayer as in the case of the metabolites. The functions of these granules have not been clearly established.

Metabolically the cells of the monolayer are very active. They exhibit a high Q_{O_2} and possess complete enzyme systems for nucleic acid degradation and for the biosynthesis of bile salts, cholesterol, urea and uric acid.

However, the monolayer's most striking biosynthetic

capabilities are related to its secretory functions. Cells in the monolayer synthesize and secrete all of the categories of digestive enzymes found in vertebrates with the exception of pepsin and thus are capable of effecting complete digestion of proteins, lipids and carbohydrates. In addition, experiments suggest that a few non-vertebrate enzymes such as cellulase and chitinase are also secreted.

Finally, the monolayer can absorb foreign materials and waste products through its basement membrane, concentrate them, and extrude them via the cells' free surfaces, thus indicating a possible excretory role.

Therefore, the monolayer may be used to study a variety of cellular functions which normally are not carried out in the same tissue. And, because of the geometric arrangement of cells into regions, cytological and cytochemical techniques are aided by the monolayer's natural orientation.

If the hypothetical monolayer is formed into a cylinder, (more properly, a long, slender cone) and then encased in a delicate network of circular and longitudinal muscle fibers, an entirely new level of investigation is possible, that of physiological control of the cellular functions discussed above. What is the relationship between muscle contraction and each of the cellular functions? By what mechanisms are the functions and the muscles controlled?

Finally, since the entire network does not contract equally, what coordinates its selective contractions?

The system described is, of course, not hypothetical but a single tubule from the crayfish hepatopancreas. Moreover, the description did not exhaust its useful properties. Its size, for instance, is about 2-4 mm. long and 0.2-0.4 mm. in diameter which is large enough for ease of isolation and study under the dissecting microscope but small enough for the compound microscope or for cutting complete cross sections for electron microscopy. The system can be studied in vitro for several days in a drop of crayfish blood at slightly lowered temperatures. Moreover, because of the hepatopancreas' location inside the crayfish and the transparency of its connective covering, little skill is required to observe individual tubules in vivo while the crayfish eats or carries on other normal activities.

Finally, the integrity of the tubule is hormonally dependent. It is sensitive to changes in the animal's molt cycle and certain cells in the tubule which are normally rich in RNA will lose this compound in the absence of hormones from glands in the eyestalk and will regain it upon addition of eyestalk extracts.

Many of the functions discussed above have never been thoroughly investigated or else were studied long before current concepts of protein synthesis and molecular biology

came into being. Most of the previous research on the hepatopancreas dealt with anatomy and histology of the gland and its various secretions but these studies can be grouped into sporadic periods over the last 125 years. For example, only one brief paper has been published on the fine structure of the crayfish hepatopancreas and this was limited to a study of the metal-containing cells.

In general, therefore, this remarkable, polyvalent system has gone largely unnoticed in spite of the current trend toward multidisciplined approaches and synthesis of biological concepts from all levels of organization.

Problem to be investigated

The versatility of the hepatopancreas allowed several choices of single processes which could be studied at many levels of biological organization. The particular process chosen for this research was the secretion of digestive enzymes.

The term secretion usually gives to the secretory act an aura of circumscription or narrowness of scope, as though it involved a simple transfer of the secretory product. In reality, secretion is influenced at many levels of biological organization. Modern concepts of secretion, such as those expounded by Junqueira and Hirsch (27) and by Gabe and Arvy (17), reveal that secretion involves at least three

general activities: (1) ingestion of raw materials by the cell from the surrounding medium; (2) synthesis and concentration of the secretory product within the cell; and (3) extrusion of the secretory product into the extracellular environment. Each activity, of course, is reflected by biochemical and morphological changes which may be studied by a variety of means. Even these three elements, broad as they are, confine the concept of secretion within the limits of the secretory cell though it is evident that secretion involves a fourth activity, transfer of the secretory product from the secretory cell to the site of action. To illustrate this, Richardson (49) has described fourteen separate factors (anatomical, neural and hormonal) which operate in mammary gland secretion after extrusion of the milk by the alveolar cells. Secretion, therefore, is the end result of factors at many levels acting in concert. These include: (1) the secretory cell's genetically determined "program" of differentiation; (2) the maze of biosynthetic pathways and their controls; (3) the dynamic activities and relationships of intracellular organelles; (4) the anatomical relationships between the secretory cell and neighboring structures which might influence it; and (5) extracellular influences such as drugs, hormones and nerve impulses, including their reception and integration as well as the mechanisms by which they influence secretion.

To limit the problem still further it was decided specifically to investigate the reported presence of secretory rhythms in the hepatopancreas. The details of previous studies on cyclic secretion of digestive enzymes will be covered in later sections but, suffice it to say, such rhythmicity has been established and it is a reflection of the cell's secretory cycle. Beyond this, no two authors agree on the exact source of the rhythmicity. It can be explained by the mode of secretion, but holocrine, apocrine and merocrine secretion have all been described in the gland. And, although sequence of secretory cell differentiation is definitely a determining factor, at least four different sequences have been described. From a practical standpoint rhythmicity is often a very desirable phenomenon. In discussing secretion of digestive enzymes, Barrington (3) mentions certain intertidal molluscs in which perfect correlation has been found between the secretory rhythm and the tidal rhythm, demonstrating how adequate digestive enzymes are economically supplied just prior to the arrival of food via the incoming tide.

Essentially, therefore, this dissertation is involved with confirming the presence of rhythmic, digestive enzyme secretion in a particular species of crayfish not previously studied, and investigating its possible sources.

Approach to the problem

Some of the work carried out in previous investigations had to be repeated with this species of crayfish, although newer techniques were utilized, in order to provide a basis for comparison. In addition, certain aspects not previously studied helped to correlate the older observations and these too were examined.

A three-fold approach was used in which data and conclusions were obtained independently in each part and then combined in a general, integrating discussion.

In Part I the morphology of hepatopancreas tubules was studied using a variety of cytological and cytochemical techniques including electron microscopy. The purpose was to gather information about the various cell types, their location in the tubule, cytochemistry, fine structure and appearance during secretion in order to clarify the mode of secretion and the sequence of cell differentiation.

In Part II the activity of one digestive enzyme, a trypsin-like protease, was assayed in hepatopancreas homogenates at close intervals for several hours after feeding and a duplicate study carried out using animals treated with colchicine to block mitosis in the tubules. The same homogenates were then assayed for protein content to study its changes and also to calculate specific activities of the protease. From the results of these studies it was possible

to determine if a secretory rhythm was present and whether or not secretion was exclusively holocrine.

In Part III it was originally intended to carry out a variety of stimulation experiments using drugs, electric shock and extracts of tissues from fed animals to study tubule contraction with regard to timing and mechanism of control. However, difficulties arose which necessitated reducing the goals of this part to simple observations in tubules under various conditions without formal experimentation.

The results of these three parts were then analyzed for either confirming or contradictory findings and then were used in conjunction with previous work in an effort to answer the central problem of rhythmic secretion and its causes.

REVIEW OF LITERATURE

General Trends in Hepatopancreas Research

Although observations on the crustacean hepatopancreas have been described in the literature for at least a century and a quarter, the total information accumulated is meager when compared to other areas of biology for several reasons: First, crustacean physiology, unlike that of the vertebrates, is a young science. For example, in the area of crustacean endocrinology, Carlisle and Knowles (4) point out that the endocrine glands of vertebrates had been described in detail many years (even centuries in some cases) before experiments on function were performed and that this knowledge facilitated the ablation and other physiological techniques used in endocrine research. In crustacean endocrinology, however, evidence for blood-borne hormones was originally based on simple physiological experiments and preceded detailed anatomical and histological descriptions by several years. Even the morphology of the sinus gland was not described until 1947. Although endocrinology is perhaps one of the physiological areas most affected by lack of precise anatomical information, other areas have been similarly delayed.

The second reason is an apparent lack of communication among scientists who were interested in the hepatopancreas,

both in related disciplines and in different countries. A literature search reveals that many times gross anatomists and histologists working on the hepatopancreas were unaware of each other's findings and that a definite information barrier existed between the earlier works of American and German investigators and, more recently, between those of German and Japanese workers. Later, it will be shown how some 70 years of valuable research has become buried in the old German literature.

A third probable factor is that digestion in the crayfish has its most dramatic aspects in the complex mechanical system for mastication, made up of chitinous ossicles, bars, plates and teeth, filters of setae and several pairs of powerful muscles, collectively referred to as the gastric mill and compared to which the greenish, soft hepatopancreas seemed uninteresting to the anatomically orientated biologists of the day.

Finally, as is the case in many fields, general interest in the hepatopancreas and its secretory functions could only occur after two prerequisites had been met. First, some imaginative and respected scientist would have to demonstrate the gland's broad potential value in acquiring new biological knowledge; and, second, a more quantitative method than those generally available to zoologists had to be developed by which secretion could be studied. The two

factors appeared almost simultaneously in the 1880's and the bulk of hepatopancreas research, sporadic though it has been, appeared after this date.

With regard to meeting the first prerequisite, this is usually attributed to the publication in 1880 of a small but unique zoology text by Thomas H. Huxley (25) which was widely read and enjoyed in several languages for its completeness and attention to detail, its literary prose which made life processes understandable, and its beautiful woodcuts and line drawings. It was a unique text because Huxley demonstrated the universality and integration of life processes by presenting all the many facets of zoology in a single animal; the latter was, of course, "the commonest and most insignificant of animals", the lowly crayfish. In chapter II, there is a poetic description of chemical digestion after which Huxley describes the sources of the digestive fluid, the "livers". This included the anatomy of the hepatopancreas, the relationship of the ducts and the "innumerable caeca", and, in one of the earliest descriptions of secretory cell differentiation in the hepatopancreas tubule, he explains that it is the cells of the tubule epithelium "which are the seat of the manufacturing process which results in the formation of the secretion" and that, "To this end they are constantly being reformed at the summits of the caeca. As they grow, they pass down

towards the duct and, at the same time, separate into their interior certain special products, among which globules of yellow fatty matter are very conspicuous." He goes on to describe the intracellular growth of these globules from a minute size in young cells near the summit of the caeca to their state in the more proximal cells where the globules are larger and more numerous and from which the globules are released and eventually removed from the gland via the duct system. He concludes, "In fact, few glands are better fitted for the study of the manner in which secretion is effected than the crayfish's liver."

In 1883, at about the same time zoologists around the world were reading Huxley's book, R. Heidenhain published a handbook of physiology which included the results of fifteen years of investigations on secretion in gastric and salivary glands. Heidenhain's work is described in detail by Babkin (2) who credits Heidenhain with having laid the foundation of our knowledge of the secretory process and of being the first to appreciate the internal changes of gland cells which are collectively referred to as the secretory cycle. Heidenhain's "method" was simply to correlate histological observations and biochemical assays of digestive enzymes in the same tissue, on the same time scale, after stimulation. This approach, although an obvious one for today's biologist, was an innovation then because it required combining

the "artistry" of the histologist and the more quantitative skills of the organic chemist, two demanding and usually exclusive abilities, in a single individual. Although similar efforts had been made before him, Heidenhain successfully brought the two fields together in studying the secretory cell and used the combination to reveal a wealth of information about many cellular processes as well as secretion.

After the appearances of Huxley's exposition of the role of the hepatopaneas and its research potential and Heidenhain's interesting approach to the study of secretion, many zoologists, especially in Germany, began to turn their attention to this organ, first examining its histology and secretions and then combining the two in the manner of Heidenhain, all of which resulted in a large mass of literature on the subject in the 50-year period from 1880 to 1930. Then, almost as suddenly as it arose, interest in the hepatopaneas waned and the gland slipped into obscurity. The latter is reflected today by the brief coverage the hepatopaneas and its multiple functions receive in modern zoology texts in spite of past interest. During the last few years, however, the gland has been studied with increasing frequency, possibly because of a revitalization of the comparative and invertebrate aspects of biology and because it appears to be an interesting system for use in

modern biological research.

Thus, research on the hepatopancreas appears to fall into five periods:

1. prior to 1880: a few isolated studies concerned mainly with anatomical considerations, especially nervous innervation and tubule musculature.
2. 1880 to 1905: accelerated research, possibly stimulated by Huxley's work, characterizing the digestive juice and its enzymes, making general histological observations of the tubule, and performing basic physiological experiments to elucidate hepatopancreatic functions (e.g. feeding and injection of dyes).
3. 1905 to 1930: increasingly sophisticated correlations between histology and physiology in the manner of Heidenhain, culminating in the work of Hirsch and Jacobs in 1928 - 1930.
4. 1930 to 1955: a blank period characterized by nearly complete lack of interest in the hepatopancreas; many of the former investigators had turned their attention to secretion in vertebrates.
5. 1955 to present: sporadic but increasingly frequent studies in which the hepatopancreas has been used to investigate a variety of topics including cyto-differentiation, the role of mitochondria in osmo-

regulation, unusual glycolytic pathways and the cytochemical effects of molting hormones.

Studies Related to Protease Secretion

A review of specific investigations relating to the cytology and physiology of protease secretion will now be presented, beginning with the digestive juice itself and then tracing its origin to the dynamic activities of the tubule epithelium.

Nature of the digestive juice

A general review of how digestive juice is collected for analysis, its enzymes, bile salts, ions and pH may be found in Vonk (56). A complete list of crustacean digestive enzymes investigated prior to 1954 was prepared by Mansour-Bek for "Tabulae Biologicae" (37).

According to Yonge (60), it was Hoppe-Selyer in 1876 who first demonstrated that the crustacean hepatopancreas was a digestive organ and, by 1910, considerable evidence had accumulated for the presence of amylolytic, proteolytic and lipolytic enzymes in the digestive juice. In the same paper, Yonge reported the presence of enzymes in the Norway lobster, Nephrops norvegicus, for the digestion of starch, glycogen, sucrose, maltose and lactose with optimum digestion at 57°C and neutral pH; for the splitting of fats and a wide range of fatty acid esters at an alkaline pH; and for

the digestion of fibrin to amino acids, also with an alkaline pH optimum and believed to be tryptic rather than peptic in nature. In 1928, Krüger and Graetz (30) found optimal protease activity at pH 7.5 and 50°C for the digestive juice of the crayfish Astacus; however, in the same year, Shinoda (51), using the same species, reported pH optima of 6.2 - 6.7 for peptones and proteoses and 8 - 9 for fibrin and gelatin. In 1932, Mansour-Bek (38), using the crab Maja squinado and a variety of substrates, compared the activities of digestive enzymes in gastric juice and crude hepatopancreas extracts with the activities of enzymes purified by column absorption techniques. Casein-splitting by the juice and crude extract was most active at pH 6.2 and, for gelatin, at pH 6.0. The purified proteinases had optima for casein and gelatin at pH 8.1 and 7.4 respectively and the purified enzyme is activated by pig enterokinase but HCN and H₂S had no effect. The study indicates a strong functional similarity between this protease and vertebrate trypsin. According to Vonk (55), no protease similar to vertebrate pepsin has been found in crustaceans or other invertebrates and, apparently, none of the crustacean digestive enzymes have been isolated or crystallized for further analysis. There may be several as yet undiscovered digestive enzymes in the hepatopancreas. In 1964, Kooiman (29)

raised the number of digestive carbohydrases in the lobster Homarus and in Astacus to 13 including chitinase and cellulase.

Transport of digestive juice

The factors involved in transporting digestive juice from its source in the hepatopancreas to its initial site of action in the alimentary canal are (1) anatomical route, (2) mechanical factors effecting a flow of the juice, and (3) physiological control of these factors.

Both Yonge (60) in 1924 and Lochhead (35) in 1950 have described a system of grooves and channels in the wall of the alimentary canal between cardiac stomach and midgut at the junctions of the hepatopancreatic ducts. The system is beset with numerous setae which act as filters and allow the passage of only very small particles. It thus forms a pathway through the complex cardiac and pyloric stomachs, including the gastric mill. The main route for partially digested food particles entering the hepatopancreas (or, in the reverse direction, for transfer of digestive juice to the alimentary canal) probably follows the ventral groove which begins at the esophageal portion of the cardiac stomach and then divides into two forks, passes on either side of the cardiopyloric valve, and proceeds through a gland filter with setae spaced 10 micra apart into channels lead-

ing to the openings of right and left divisions of the hepatopancreas. All along the route there are muscular valves which regulate flow by closing the grooves.

In regard to mechanical factors which effect a flow of digestive juice, most of the modern literature (that of the last 40-plus years) is practically silent on the subject. Ramsay (46), in 1952, published a monograph on invertebrate physiology in which he explains that molluscs and crustaceans use different mechanisms to remove digestive juice from the tubules. In molluscs, transport occurs through ciliary movement along the tubule epithelium but, in crustaceans, the epithelium is not ciliated and secretions are transported by contractions of a "thin coat of muscle" surrounding the hepatopancreas tubule. The monograph, however, does not include a bibliography and so the statement cannot be traced in the literature. In 1959, Ogura (43) included some hepatopancreas morphology in his paper on metal granules in the tubule and shows a line drawing of a tubule encased by circular and longitudinal muscle fibers. However, the muscles are not discussed in the paper and, again, references are not given. By chance, this author came across an unpublished report prepared by Smith (52) in 1965 for an undergraduate research course. The paper was concerned with the system of muscle fibers in the hepatopancreas tubule and included an excellent bibliography.

From the latter, this author commenced a literature search which revealed that a mass of research had been carried out on the muscle network during the period 1845 to 1914. The last and most complete work was by Pump (45) in 1914 who surveyed the literature of this 70-year period in addition to making his own observations. The muscle net consists of two types of fibers; thick, striated, circular fibers, containing one nucleus per fiber, and evenly spaced along the length of the tubule except at the distal tip where they are spaced at smaller intervals; and very fine, usually non-striated longitudinal fibers which appear to connect two successive circular fibers, and which come off the latter at all angles and sometimes divide into two or three branches. The network appeared to lie on the surface of a tunica propria which enclosed the tubule itself. Apparently all of the work done during this period was descriptive histology and morphology with no physiological investigations. In regard to the striations, Pump noted only A and Z bands in the circular muscles but Smith (52), using Biebrich's scarlet, was able to distinguish A, I, H, and Z regions in whole mounts.

In spite of the apparent lack of physiological experiments, the obvious anatomical relationship of the muscle network to the tubule epithelium led Yonge (60) to write: "The forcing out of the secretion and taking in of the dis-

solved matter is brought about by a rhythmical contraction and expansion of the hepatopancreatic tubules. The circular muscle fibers cause contraction, and the relaxation of these together with the contraction of the striped longitudinal fibers cause expansion." The latter mechanism is difficult to conceive.

In regard to physiological control of the tubule contractions, neither hormonal nor neural mechanisms have been demonstrated in crayfish. Several studies have been carried out on the neural control of crayfish intestinal movements. but nothing pertaining to the hepatopancreas. Even the question of innervation has not been answered. Neuro-physiologists cite only three basic references which even mention innervation of the hepatopancreas, the most detailed of which is by Keim (28) in 1915. In the crayfish, the two large circumesophageal commissures of the ventral nerve cord each give off a delicate, medial branch and these unite anterior to the esophagus forming the stomatogastric nerve. This nerve is then closely applied to the anteroventral surface of the cardiac stomach and follows its curvature first forward, then upward and, finally, backward on the stomach's dorsal surface. At the point where the nerve passes between the anterior gastrics, two large muscle bundles which attach the cardiac stomach to the dorsal carapace, it forms the ventricular ganglion and then continues

posteriorly as the dorsal ventricular nerve, passing off paired right and left branches along the way. Near the region of junction between cardiac and pyloric portions of the stomach, the nerve divides into right and left branches, each of which turns outward and downward abruptly, giving off more branches as it follows the curvature of the alimentary canal ventrally. One of these small branches, which Keim calls the liver nerve, can be traced to the junction of midgut and hepatopancreatic duct. At this point, however, the nerve ramifies to such an extent that it cannot be traced beyond the junction, thus leaving open the question of innervation. Keim quotes an earlier German investigator, Schlemm who, writing in Latin, described the same nerve (and the same difficulty) in 1844.

Cytology of the hepatopancreas tubule

In 1880, Huxley (25) described the individual tubules as having an outer wall lined by a single layer of epithelium. In the same year, Weber (57), who also described the muscle network, identified two cell types in the epithelium of tubules from Astacus fluviatilis, the enzyme-producing Fermentzellen and the fat-storing Leberzellen. The same two types were observed by Frenzel (15) in 1893 who called Fermentzellen, those which possessed a single, large vacuole, and Fettezellen, those which had many lipid droplets.

Cytoplasm of the enzyme cells had a fibrillar appearance when stained with either light green and safranin or hematoxylin and safranin. In addition, he observed a third cell type, the enzyme-replacement cells, which also had a fibrillar cytoplasm but whose vacuoles had a range of sizes down to the limits of resolution. Mitosis of enzyme- and fat-mother cells was observed in the blind tip of the tubule but the two cell types could also form amitotically from pre-existing cells of the same type. A striated boarder was present on the enzyme, fat and replacement cells.

In the United States in 1928, Dorman (7), in his studies on the comparative physiology of digestion, described the morphology and histology of the hepatopancreas. He, too, described two principle cell types which he names hepatic and pancreatic cells. The first was a columnar epithelial cell possessing one or two round and basally situated nuclei and with granular cytoplasm containing pigmented inclusions, fat globules and glycogen deposits; pancreatic cells stained much more darkly than hepatic cells with most cytoplasmic stains, had a deeply granular appearance, and their long axes were perpendicular to the basement membrane. The basal portion was broad and rested flat against the basement membrane but the apical end was attenuated and drawn out to form a duct and was closely applied to a crypt in the lumen. Occasionally the ducts of several pancreatic

cells coalesced before meeting a crypt.

The most extensive cytological description of the tubule epithelium was made by Jacobs (26) in 1928 who studied the formation of secretory granules in the hepatopancreas of Astacus leptodactylus. Jacobs described four cell types which he called Embryonale zellen (E-cells), Restzellen (R-cells), Fibrillenzellen (F-cells) and Blasenzellen (B-cells). A fifth type, Wanderzellen, was not involved in the secretory function of the gland. E-cells were undifferentiated columnar epithelial cells which, together with mitotic divisions, were restricted to the blind, distal tip of the tubule. More proximally other E-cells were found with evidences of beginning differentiation such as a basophilic vacuole, the parasome, closely applied to the apical surface of a large elongated nucleus, and a few, discrete Golgi apparati. R-cells and F-cells were found throughout the length of the tubule except for the tip. R-cells were absorptive in appearance and distinguished by the presence of several fat vacuoles and a small, rounded nucleus in the basal cytoplasm. F-cells had a larger nucleus which was more centrally located. Most of their cytoplasm was richly basophilic and had a fibrillar appearance except for a region in the basal portion which was acidophilic and non-fibrillar. Both cell types possessed a striated border.

B-cells were the most striking of the four due to the

presence of a single, large, blister-like, secretory vacuole which left only a small amount of cytoplasm at the apical and basal ends of the cell. The nucleus was pressed flat against the basal membrane by the vacuole. B-cells were restricted to the middle 50-60% of the tubule and their striking presence allowed Jacobs to divide the tubule into three regions: Region I was the most distal portion including the tip and contained E-cells followed by R- and F-cells; Region II stood out due to the presence of B-cells, although R- and F-cells were located here also; Region III, the most proximal portion, resembled the epithelium of the collecting ducts and contained, in addition to R- and F-cells, some degenerating cells with pycnotic nuclei. B-cells also possessed a definite, striated border.

Jacobs also described intermediate cell types, especially in Region I; for example, E-cells with increasing amounts of lipid or increasing basophilic and fibrillar cytoplasm, and F-cells with a single vacuole of increasing size.

More recent authors have described similar cell types. In 1957, Travis (53) found that in the spiny lobster, Panulirus, the absorption cells, under certain conditions, had large concentrations of alkaline phosphatase in the striated border, calcium phosphate deposits or calcospherites in the apical cytoplasm, and large amounts of gly-

cogen and fats. In the crayfish Procambarus clarkii Ogura (43), in 1959, observed the presence of iron granules in the fibrillar cells and copper granules in the absorptive cells and thus designated the two types Fe-cells and Cu-cells respectively. Miyawaki et al. (40), in 1961, confirmed these results in the same species and added the following observations. The Fe-cells were rich in RNA with the exception of the Fe-containing vacuole itself. The latter was rich in mucopolysaccharides or mucoproteins bound with iron. The cytoplasm of Fe-cells was also rich in phosphatases and calcium. However, the Cu-cells contained copper only when this metal was in excessive concentrations in the media. In another study, in 1962, Miyawaki and Tanoue (42) presented what is probably the only previous electron micrographic study of cell types in the crayfish hepatopancreas albeit quite brief and concentrating on the metal-containing cells. The Fe-cells possessed an extensive endoplasmic reticulum and a very large, electron-dense iron granule. The Cu-cells possessed a large, complex organelle which was identified as the Cu-granule. The organelle was bounded by a double membrane, with many internal vesicles, membranes and electron-dense particles, irregular in shape, size and distribution. Both cell types possessed a border of microvilli.

Secretion and cell differentiation

In addition to the morphological descriptions given above, Jacobs (26) also observed the growth and maturation of Golgi-associated secretion granules in F-cells with gradual coalescence of the granules until a secretory or B-cell resulted. On this basis, and because of the location and intermediate forms of the four basic cell types, he postulated the following sequence of cell differentiation: mitosis, E-cell, R-cell, F-cell, B-cell, secretion; however, differences in morphology cast some doubt about including R-cells in direct sequence with the other cell types. This sequence would account for the various locations of the cell types and it provided a cytological confirmation of Huxley's original observation that the secretory vacuoles grew in size as cells migrated from the distal end of the tubule to the more proximal region where secretion took place.

In the period 1928-1930, Hirsch and Jacobs (21, 22) and Hirsch and Buchmann (20) published a classical series of investigations in which the subjects of digestive enzyme secretion and cell differentiation were studied in the crayfish Astacus leptodactylus. The studies included enzyme assays and cell counts (21), correlation and analysis of the results (22)', and cytochemical studies of the various cell types (20). Activities of amylase, casein-protease, and

peroxidase were assayed in the stomach juice and in glycerin extracts of the hepatopancreas in starved crayfish and in animals sacrificed every half-hour after feeding for $6\frac{1}{2}$ hours. Histological preparations were made from the same glands and counts were made of mitotic figures and each of the four cell types in the tubule. The results indicated that all of the enzyme activities fluctuated with time according to a regular rhythm. The rhythms of amylase and protease were in agreement while that of peroxidase was different. The curve for each digestive enzyme activity possessed two maxima in the $6\frac{1}{2}$ hour period. Moreover, the cycling in the stomach juice, although similar to that of the extract, followed it by $\frac{1}{2}$ to 2 hours depending on the enzyme and thus confirmed the source of the digestive enzymes in the stomach juice to be the hepatopancreas. These results were reproducible in all seasons of the year although the over-all values and maxima were highest in Spring. When curves of cell counts were compared with those of enzyme activities, the former also varied rhythmically, but only the B-cells were in phase with enzyme activity in the gland extract. This was taken as evidence that the source of the enzyme was the B-cell, a conclusion previously made on purely morphological grounds.

When the other curves were carefully analyzed, it was apparent that the cycles for the various cell types, in-

cluding dividing cells, were all slightly out of phase so that a sequence of maxima was repeated nearly three times in a $6\frac{1}{2}$ hour period. This sequence, it was concluded, represented the sequence of cell differentiation leading to secretion. The sequence was: mitosis, E-cells, F-cells, B₁-cells, B₂-cells, B-cells, holocrine secretion. R-cells were differentiated separately from E-cells, and B₁ and B₂ were stages in the maturation of the B-cell. The time required for differentiation of E-cell to F-cell was 2 hours, for F-cell to B-cell differentiation 1 hour, and for movement of the enzyme to the gastric juice 0.5 to 1.5 hours. The first complete cycle (utilizing cells which divided prior to feeding and thus called the hunger phase) took place during the period 0 to 4 hours post-feeding; the second cycle (restitution phase #1) in the period 1.5 to 6 hours; and the third cycle (restitution phase #2) began at 3.5 hours but its completion was not covered in the time period studied. Each succeeding phase required a slightly longer period to complete as the lapse of time since stimulation increased.

To explain Region III, Hirsch and Jacobs postulated a secondary flow of cells from the duct which also contributed to the secretory cells in Region II.

The cell count method (but not the enzyme assays) was also used by van Weel (54) in 1955 in studying the hepato-

pancreas of the freshwater crab, Atya spinipes, over increasingly longer intervals of time (30 minutes to 6 hours) for 36 hours after feeding. He, too, observed cycling of the cell numbers but the mode of secretion observed in sectioned material was merocrine rather than holocrine as reported for Astacus. This was confirmed by the observation that, in living cells, a slight pressure on the coverslip resulted in extrusion of secretory material without rupturing the cell membrane. The author postulated cyclic cell secretion followed by restitution of secretory material in the same cells. The cell types he described are similar to those in Astacus but different names are used to emphasize the cyclic nature of their differentiation. The sequence of differentiation he reported was: embryonic cells, transitional cells (T.C.), light cells (L.C.) which were similar to R-cells, extrusion cells (E.C.) which were secretory cells, merocrine secretion, empty cells, dark cells (D.C.) which were similar to F-cells, and reformation of the light cells. Van Weel considered the fibrillar appearance of the dark cells an artifact caused by protein precipitating fixatives. In his scheme, replacement light cells are formed from embryonic via transitional cells when the mature cells were lost or worn out.

Travis (53) reported apocrine secretion in the hepatopancreas of Panulirus but no attempt was made to interpret

this mode of secretion in terms of cell differentiation or the secretory cycle.

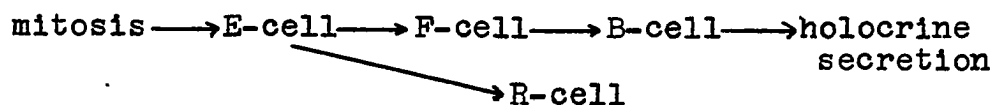
In studying the various metal granules in the hepatopancreas tubule cells, Ogura (43) in 1959 was able to trace all three cell types (Fe-cell, Cu-cell and B-cell) back to the distal tip of the tubule on the basis of their specific inclusions. Fe-cells are labelled by their rich basophilia and by the presence of iron, either as the granule or, in immature cells, as diffuse iron in the cytoplasm. Cu-cells (i.e. absorptive cells) accumulate fat droplets very early in their development and B-cells have neither the fat droplets nor the iron granules. Since he believed exchange or ready release of the metal granules was unlikely, the three types must each arise independently from E-cells. He also concluded that the secretion in Region II is holocrine and that Region III does not result from a secondary flow of cells from the duct but a continuation of the flow from the distal end minus the secretory cells lost in holocrine secretion.

Finally, in 1964, Davis and Burnett (6) combined cytochemical staining and autoradiography to investigate cell differentiation in the hepatopancreas tubule. Tritiated thymidine was injected into specimens of Procambarus blandingii and the label appeared in the nuclei of embryonic cells after 5 minutes, of absorptive cells after 2 hours, of

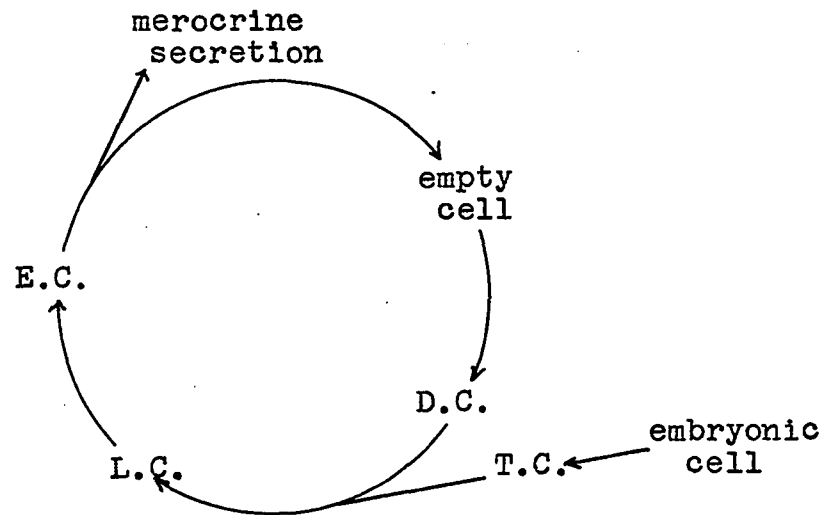
secretory cells after 24 hours, of fibrillar cells after 2 days, of proximal cells after 5 days and of cells in the main duct after 15 minutes. Tritiated uridine appeared in none of the cells after one hour and in the nuclei of all cells after 10 hours. Feeding did not alter the results. From these observations the authors concluded that the mitotic cycle in crayfish hepatopancreas is of the order of 3 to 5 hours and from the appearances of the label in the cell types, the sequence of differentiation is: embryonic cells, absorption cells, secretory cells, fibrillar cells, proximal cells.

Thus, cell secretion is reported to be rhythmic in one species of crayfish and in one crab but the postulated hypothetical causes are mutually exclusive, viz. linear differentiation and holocrine secretion vs. cyclic restitution and merocrine secretion. Apocrine secretion is reported for one species of lobster. Four different sequences of cell differentiation have been described in four hepatopancreas tubules whose cell types appear quite similar if not identical. The four postulated sequences of differentiation are summarized below:

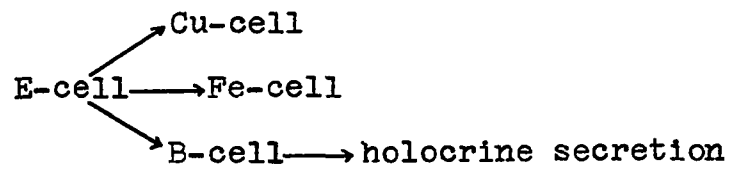
Hirsch and Jacobs (22)



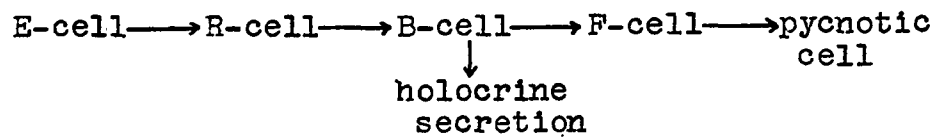
van Weel (54)



Ogura (43)



Davis and Burnett (6)



PART I. MICROSCOPIC ANATOMY OF HEPATOPANCREAS TUBULE

Introduction

In order to investigate rhythmic digestive enzyme secretion in Orconectes virilis, a crayfish not previously used for this purpose, it was first necessary to study the cytology of its hepatopancreas tubule epithelium for the following reasons: first, to compare the cell types of Orconectes with Astacus and Procambarus which have already been described; second, to apply some of the newer cytochemical techniques to the tubules at intervals after feeding in the manner of Hirsch and Jacobs (21) but which were not yet available when these authors studied the problem; third, to fully characterize E-, R-, F- and B-cells in Orconectes using light microscopy and cytochemistry and then to study the same cells with electron microscopy and correlate the results; and, finally, to study the relationships between the network of muscle fibers surrounding the tubule and the epithelial cells within the tubule.

Methods of Procedure

Intermolt specimens of Orconectes virilis averaging about four inches in length were obtained in late winter from the E. G. Steinhilber Co., Oshkosh, Wisconsin. The crayfish were maintained in shallow, artificial spring water made up according to the formula of Hopkins and Pace (23)

but omitting FeCl_3 due to its precipitating action. They were kept at room temperature, 20-25°C, and fed either raw liver or hamburger twice a week. For most experiments the animals were fasted two days prior to being sacrificed.

The general procedure for collecting tissues is as follows, with some modification for particular techniques. Hepatopancreas tubules were obtained by first removing the entire dorsal portion of the carapace with sharp scissors and probe, exposing internal organs in the cephalothorax. Anterior and posterior lobes of right and left glands were then gently pulled from their respective spaces between other organs using a dull probe. The digestive tract was then severed at the esophagus and at the beginning of the hindgut thus freeing the hepatopancreas, stomach and midgut from the other viscera. The entire hepatopancreas was removed by picking up the stomach and midgut, to which the glands were attached. The organs were transferred to a petri dish with van Harreveld's solution, a balanced physiological saline solution for crayfish, which was made up according to the formula given by Welsch and Smith (58). Under a dissecting microscope, the connective tissue covering was removed with forceps allowing the tubules to float free in the media although still attached at their proximal ends to the ducts. The glands were then divided into small pieces, each containing 20 to 30 tubules, and washed in two

changes of ringers. For some studies, individual tubules were isolated using watchmaker's forceps and microdissection instruments made from insect pins and finely drawn glass rod.

Light microscopy

Serial sections After carapace removal the internal organs were flushed with 2.5% veronal-buffered glutaraldehyde at pH 7.8, the glands transferred into fresh glutaraldehyde and all further dissection performed in this fixative with periodic changes. Tubules were exposed to glutaraldehyde for a total of 60 minutes. The isolated tubules were then washed in veronal buffer for some 30 minutes. Some were used for electron microscopy (see below) and others were post-fixed in 1:3 acetic-alcohol for 30 minutes. (The latter was found to extract lipids which interfered in the PAS reactions without appreciably altering cell morphology). Tubules were dehydrated in an alcohol series to benzene and individually embedded in paraffin. Serial cross sections of entire tubules were cut at 8 micra and mounted on albuminized slides, four sections to a slide. The following three staining techniques were alternated on succeeding slides.

Proteins Mercuric brom phenol blue technique of Mazia, Brewer and Alfert (39) Sections were rehydrated to 95% ethanol, stained for 15 minutes in a solution of

Brom phenol blue and mercuric chloride in 95% ethanol, rinsed for 20 minutes in 0.5% acetic acid, and differentiated in acidified water (pH 6-7 for 3 minutes to bring out the blue color). Sections were then dehydrated and mounted in HSR media.

DNA polysaccharides and protein Triple stain of Himes and Moriber (19) Sections were rehydrated to water, hydrolyzed 12 minutes in 1 N HCl at 60°C, rinsed in water, stained 5 minutes in Azure A-Schiff for DNA, rinsed, bleached and rinsed twice, oxidized in periodic acid for 2 minutes, rinsed and treated with basic fuchsin-Schiff for polysaccharides, bleached and rinsed twice, rinsed and stained in Naphthol yellow for proteins, rinsed and dehydrated in tertiary butyl alcohol, and mounted in HSR.

DNA and RNA Azure B bromide method of Flax and Himes (14) Dehydrated sections were stained three hours in buffered Azure B bromide, pH 4.0, at 40°C, rinsed in ice water, blotted, rinsed, differentiated in tertiary butyl alcohol overnight, cleared in xylene and mounted in HSR.

Changes in osmiophilia after feeding Crayfish were starved two weeks at the end of which time some were fed raw liver. Glands were collected from the starved animals and from fed animals at half-hour intervals after feeding. Pieces of hepatopaneas containing 3-6 tubules were treated with the Ludford osmium impregnation method for Golgi appa-

ratus as described in Humason (24). Tubules were fixed in Mann's osmic sublimate (0.5% osmium tetroxide, mercuric chloride and sodium chloride in water) for 18 hours, washed 30 minutes in distilled water, and impregnated with osmium tetroxide according to the following schedule: 2% for three days at 30°C, 2% for 1 day at 35°C, 1% for 1 day at 35°C, and 0.5% for 1 day at 35°C. Tissues were then washed in distilled water 1 day, dehydrated, cleared in benzene, embedded in paraffin and sectioned at 10 micra. The original procedure stated 6-7 micra for Golgi observations but the osmium impregnation yielded very "gristly" sections due to the high lipid concentration in the hepatopaneas and sections thinner than 10 micra could not be cut. Sections were mounted on albuminized slides, deparaffinized and mounted in HSR.

Muscle fibers Richardson's myoepithelium stain (49), designed for mammary gland, was modified slightly for use with hepatopaneas. Pieces of gland containing 3-6 tubules were fixed four to six weeks in Weber's fixative (dioxane, isopropyl alcohol, formalin, formic acid, cobalt nitrate, chloral hydrate and glacial acetic acid), washed in running water overnight, washed several hours in sodium acetate buffer at pH 5.3 and then treated with a silver reagent (silver nitrate, ethanol, pyridine and water) for 10 minutes at 55°C. Tissues were then rinsed for five sec-

onds in absolute ethanol and reduced in a solution of hydroquinone and formalin in water until a dark brown color appeared. They were then washed in distilled water, fixed in 5% aqueous hypo and dehydrated in tertiary butyl alcohol. Some tissues were paraffin embedded and sectioned at 20 micra but the majority were prepared as whole mounts. For the latter, broken cover slips were used as spacers and the tubules were mounted in HSR.

Nerve fibers Two techniques were utilized to look for nerve fibers in hepatopancreas tubules.

In situ staining with leuco-methylene blue The method of Larimer and Ashby (34) called for 0.4% methylene blue, reduced to the leuco-form with 0.01 M sodium hydro-sulfite, both solutions in van Harreveld's solution. Tissue was immersed in the colorless stain for twenty minutes, transferred to fresh media for oxidation to the colored state, and the color enhanced with 1% hydrogen peroxide in the crayfish perfusion fluid. Some tissues were prepared as whole mounts after dehydration in tertiary butyl alcohol.

Double-impregnation silver staining Fitzgerald's technique (13) required fixation in a mixture of alcohol-picric acid, formaldehyde and glacial acetic acid for three days, followed by treatment in absolute alcohol for 24 hours, clearing in benzene, embedding in paraffin and sectioning at 15 micra. Sections were mounted on albu-

minized slides and stored overnight at 37°C. Sections were then deparaffinized and brought to water within 30 minutes, treated with 10% silver nitrate for 2 hours at 56°C, washed in three changes of distilled water in 1.5 minutes, and stored in 0.2% protargol-S solution for 18 hours at 37°C. They were then rinsed, developed in hydroquinone-sulfite for 5 minutes, washed in running tap water, rinsed in distilled water, treated with 0.1% acidified gold chloride solution for 10 minutes, washed for 0.5 minute, developed in aniline-alcohol for 30 seconds, washed for two minutes, fixed in sodium thiosulfate for 10 minutes, dehydrated, cleared and mounted.

Living tubules In addition to the above techniques, phase microscopy was used to observe tubules in a variety of states including both fresh specimens and tubules which had been maintained in vitro in a hanging drop of crayfish blood for 1 to 5 days.

Electron microscopy

Tubules were obtained by first flushing the internal organs with glutaraldehyde and then dissecting in this fixative in the same manner as described under "Light microscopy, Serial sections". After being washed in veronal buffer, tubules were postfixed in veronal-buffered 1% osmium tetroxide at pH 7.8. This was followed by dehydration in an

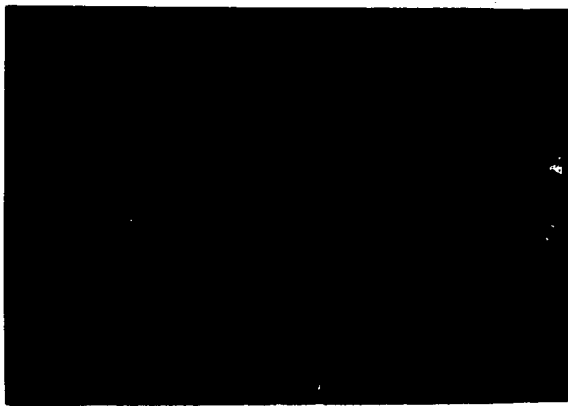
acetone series and flat-embedding in Maraglas using aluminum boats. Alternate embedding methods such as empty capsules and capsules with drilled holes in polymerized plastic were not satisfactory. The above method allowed optimum orientation during block trimming, and ease in choosing particular regions along the tubule for sectioning.

Cross sections were obtained from several locations along the length of the tubule such as the distal tip, 0.1-0.5 mm. and 0.75 mm. from the tip, and the middle of the tubule to insure sections through a variety of cell types. Sections were cut on either a Porter-Blum microtome or LKB ultratome using a glass or diamond knife respectively. They were mounted on parlodion-coated grids and either left unstained, stained with Reynold's lead citrate (47) alone, or lead citrate followed by uranyl acetate. Specimens were examined on an RCA EMU-2A or EMU-3F instrument.

Results

The anatomy of the hepatopancreas in O. virilis (Figs. 1 and 2) is similar to that described for P. clarkii (43) and P. blandingii (6). A short, main duct arises ventrally from each side of the digestive tract at the junction of stomach and midgut, and divides into three collecting ducts. Each of these ramifies further and gives rise to hundreds of blind tubules grouped into three lobes of different size and

- Fig. 1. (top) Orconectes virilis
Species of crayfish used for most of the studies
presented in this dissertation. (x0.5)
- Fig. 2. (center, left) Partial dissection of Orconectes
virilis
Carapace has been removed exposing internal
organs. Middle lobe of right hepatopaneas
(arrow) is visible between gills (right) and
heart and gonads (left). (x0.75)
- Fig. 3. (center, right) Isolated hepatopaneas
Dorsal view of intact gland shows anterior (A),
middle (M), and posterior (P) lobes. (x1.5)
- Fig. 4. (bottom, left) Isolated hepatopaneas
Connective tissue covering has been removed from
three lobes. (x1.5)
- Fig. 5. (bottom, right) Left posterior lobe of isolated
gland
Removal of connective tissue covering allows
visualization of single tubules (arrow). (x3)



appearance. The anterior lobe is elongated with a blunt anterior end and makes up about 30% of the length of the entire gland, the middle lobe is small and rounded, while the posterior lobe is long, about 50% or more of the gland, and has its posterior end pointed. When the entire gland is removed intact it has the appearance of a butterfly with long, narrow wings (Figs. 3 and 4). Each gland is enclosed in a connective tissue sac (Figs. 4 and 5) to which the distal ends of the individual tubules are attached. This covering is continuous between right and left glands under the region of the main ducts and this bridge, rather than the fragile ducts, supports the connection of gland to mid-gut.

Light microscopy

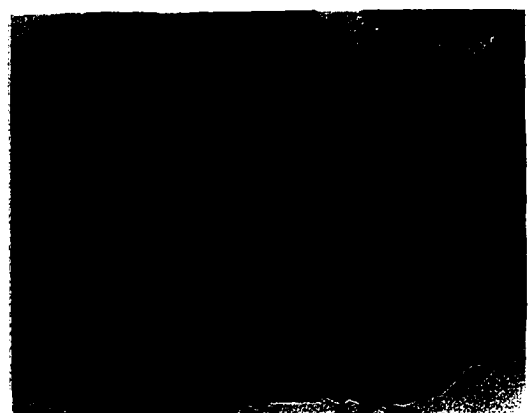
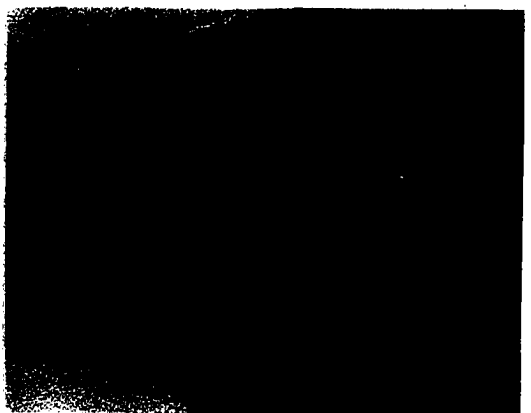
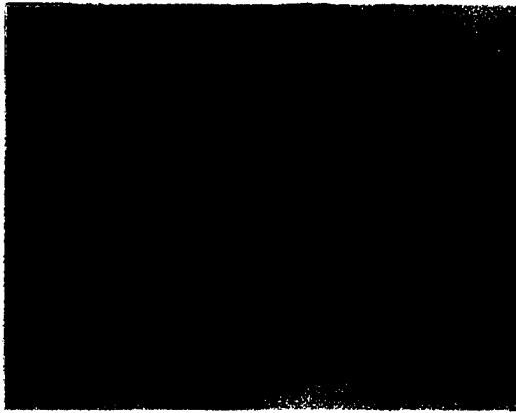
Serial cross sections through a typical 2 mm. hepatopancreas tubule yielded 256, 8-micra sections. The first 25 sections from the distal end contained undifferentiated cells and transitional columnar epithelium. The following 30 to 40 sections showed a nearly simultaneous appearance of absorption and fibrillar cells. Sections 75 through 200 contained a mixture of absorption, fibrillar and secretory cells, the last type possessing a large secretory vacuole and granules from about sections 100 to 175. In the most proximal end of the tubule the secretory cells become flat-

tened and their nuclei pycnotic. They finally disappear altogether leaving mainly fibrillar and absorption cells.

The Azure A triple stain (Fig. 6) yielded blue to blue-green nuclei and yellow to orange cytoplasm. Nuclei of connective tissue cells and muscle cells were blue throughout the tubule as were the epithelial cells in the distal portion, while nuclei of the secretory cells were a definite green. The cytoplasm of absorption and fibrillar cells also differed; fibrillar cells were darker but less reddish, except for a central vacuole, while the absorption cells were granular and reddish. The most striking sections were those containing secretory cells with large granules in the secretory vacuole. These granules were strongly PAS-positive, staining a deep red (Fig. 7). In the same cells, a short segment of the apical cytoplasm separating secretory vacuole from lumen was also PAS-positive but the portion to either side of this segment was less reddish and matched the orange of the fibrillar cells. A brush border is present on the secretory cells and, in some cases, there are faint, radiating lines of PAS-positive material extending for a short distance from brush border into the lumen (Fig. 7). Masses of material moving into the lumen from ruptured secretory cells and other evidences of classical holocrine secretion were negligible compared to that for merocrine secretion.

Azure B bromide stained the nuclei and cytoplasm of

- Fig. 6. (top, left) Azure A triple stain, distal region
Section no. 32. Fibrillar cells are darker
showing increased protein. Absorption cells are
granular; reddish color indicates PAS-positive
material is contained in these cells. (x683)
- Fig. 7. (top, right) Azure A triple stain, middle region
Section no. 104. PAS-positive granules in secre-
tory vacuoles. One secretory cell is seen re-
leasing material through a break in the membrane
while another has faint, radiating lines of
material extending outward from the apical sur-
face with no break in the membrane. (x342)
- Fig. 8. (center, left) Azure B bromide stain, distal
region
Section no. 34. Fibrillar cells stain more
darkly than absorption cells. (x342)
- Fig. 9. (center, right) Azure B bromide stain, middle
region
Section no. 108. Material in secretory vacuole
is refractile to stain. Para-vacuolar cytoplasm
stains similar to fibrillar cells. (x342)
- Fig. 10. (bottom, left) Higher magnification of section in
Fig. 8
Green granules appear blue in copies of original
35 mm. transparencies. (x683)
- Fig. 11. (bottom, right) Mercuric brom phenol blue stain,
middle region
Granules in secretory vacuole are protein-
positive. (x546)



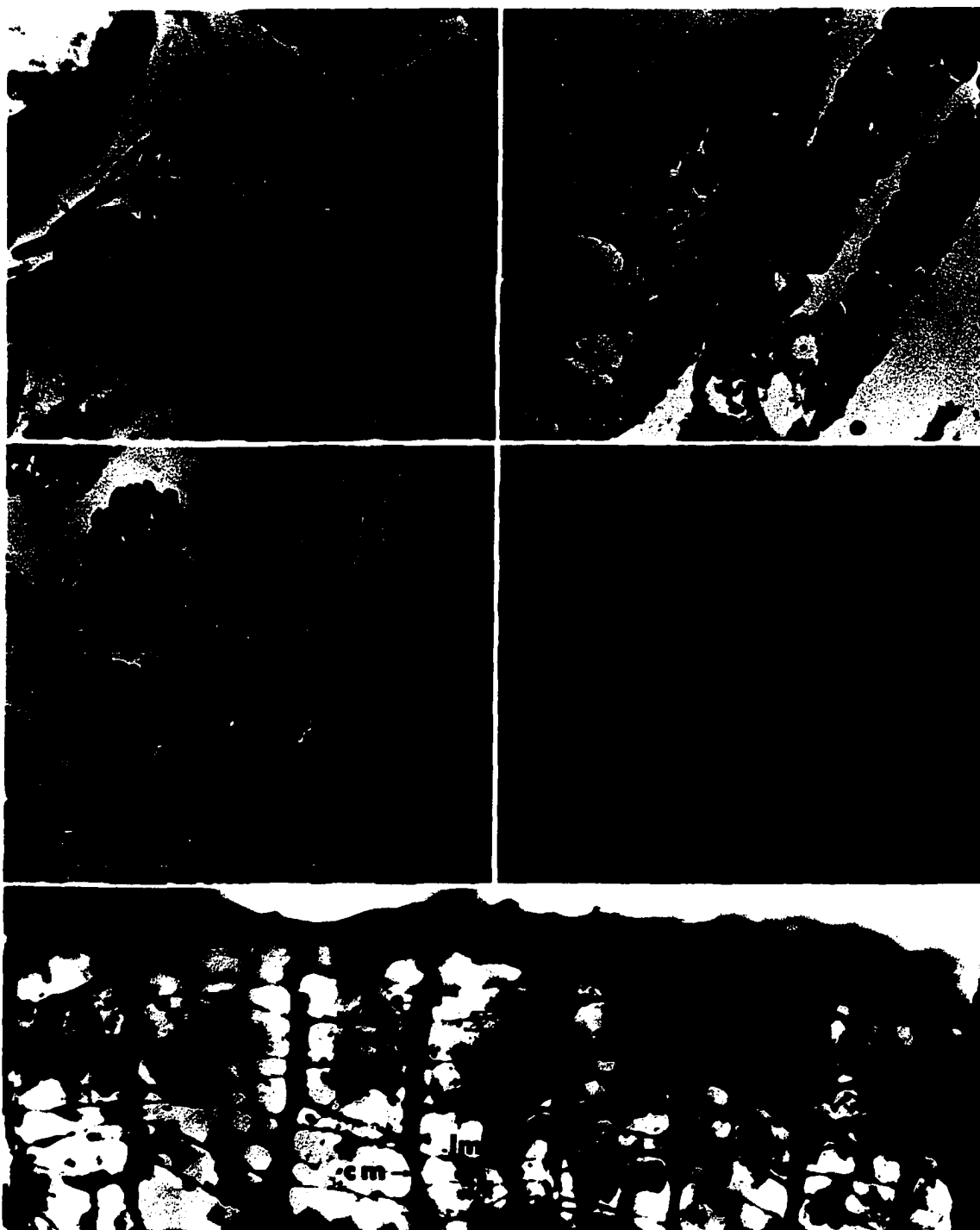
all cells blue; however, the intensity and hue varied greatly. The cytoplasm of fibrillar cells (Fig. 8) stained a very intense blue, especially toward the middle of the tubule, and stood out markedly from the other cells, while the nuclei of these cells stained blue-green. The cytoplasm of absorption cells throughout the tubule and of fibrillar cells near the distal tip stained a lighter blue or purple. In secretory cells (Fig. 9) the contents of the vacuole did not stain but the cytoplasm on either side of the vacuole stained an intense blue; however, in most cases it was difficult to differentiate between para-vacuolar cytoplasm and adjacent fibrillar cells. Many of the absorption cells, especially those in the most distal third of the tubule (Fig. 10), possessed a peculiar vacuole not seen elsewhere. Inside the vacuole was a worm-like granule, resembling a thick, twisted strand, which always stained green and appeared to be solid or crystalline in consistency.

Mercuric brom phenol blue staining also yielded blue cells; however, the intensity was lighter and the color more uniform and less bright than in the Azure B bromide sections. In spite of this uniformity, there were differences in intensity, fibrillar cells appearing more dense than the other cells and the large granules in the secretory cell vacuoles very darkly stained (Fig. 11). Embryonic and transitional cells at the blind tip and cells in

the most proximal portion of the tubule appeared less dense than the other cells in the tubule giving them a "washed out" appearance. The green structures appeared in cells prepared with this stain also.

Osmium impregnation was not successful in regard to localization of Golgi apparatus due to the large lipid concentrations in the hepatopancreas and the thickness of the sections. However, the following information was obtained from this study: First, a coiled, membranous structure was seen inside the lumen in the distal region of some tubules. In one tubule, examined three hours after feeding, the structure is continuous with the apical cell membranes and is actually being torn from the cells (Fig. 12). Second, although a few instances of what appears to be holocrine secretion are evident, there are many more examples of membrane-enclosed globules of cytoplasm either being pushed into the lumen or appearing free in the lumen indicating apocrine secretion (Fig. 13). Third, some fibrillar cells possess a vacuole located apical to the nucleus and which is non-osmiophilic and is delimited by the surrounding osmiophilic cytoplasm. Inside the vacuole is a smaller structure which did take up osmium. Fourth, at $1\frac{1}{2}$ hours after feeding, minute spheres appear in the secretory cells. Finally, there is a gradation of lipid deposit along the length of the tubule and a change in the manner of deposi-

- Fig. 12. (top, left) Intralumenal membrane
Membrane (arrow) appears to be tearing away
from apical portion of cells. In other sec-
tions the structure is tightly coiled and lies
in center of lumen. Osmium impregnation.
(x667)
- Fig. 13. (top, right) Apocrine secretion
 $1\frac{1}{2}$ hours after feeding lumen is filled with
membrane-bound droplets of cytoplasm (arrow).
Osmium impregnation. (x133)
- Fig. 14. (center, left) Lipid deposits in distal region
Heavy lipid deposits in apical and lateral
portions of absorption cells. Osmium impreg-
nation. (x167)
- Fig. 15. (center, right) Lipid deposits in proximal region
Lipid deposits are in droplet form. Size de-
creases toward proximal end of tubule. Osmium
impregnation. (x167)
- Fig. 16. (bottom) Muscle network
Large, evenly-spaced circular muscle fibers
(cm) are connected by fine, almost randomly-
arranged, longitudinal fibers (lm). Myo-
epithelium stain. (x521)



tion. At the distal end, the absorption cells are densely stained with osmium at their absorbing surfaces and along their intercellular surfaces, while the inner cytoplasm and basal end of the cells is relatively free of osmium (Fig. 14). More proximally the lipids take the form of large droplets which gradually become distributed throughout the cells (Fig. 15). Finally, in the most proximal portion of the tubule, the lipid droplets become very small in size and number.

Richardson's myoepithelium stain yielded dark brown to black tubules which, in most cases, were too dense to use as whole mounts even after treatment with weak ammonium hydroxide to dissolve excess silver or clearing in tertiary butyl alcohol. In a few tubules, however, discrete regions were found which were relatively free of lipids and provided a clear view of the muscle fibers under high power magnification (Fig. 16). Large circular fibers occurred regularly along the length of the tubule and appeared to be connected by very fine longitudinal fibers. These fibers stained intensely black against a brown background, the intensity of the latter depending on the number and proximity of lipid droplets in the region. Both circular and longitudinal fibers were covered with very fine, short projections or extensions to the underlying cells of the epithelium. It could not be ascertained whether these represented real

structures or silver deposits along the basal, intercellular junctions of the epithelial cells. Thick sections gave about the same picture except that striations could be observed in the circular fibers. The two fiber types could also be seen in freshly stained whole tubules, with difficulty, using reflected light and a dissecting microscope. The silver impregnated muscle fibers appeared bright gold against the brownish tubule surface.

Both nerve stains yielded negative results. In situ staining with leuco-methylene blue did not visualize nerve fibers but the cytoplasm of the epithelial cells stained nicely. Double impregnation silver staining gave positive results with large nerves around the stomach but within the hepatopaneas only reticular connective tissue located in some of the inter-tubule spaces were stained. It should be mentioned that this technique gave beautiful cell preservation and morphology although lack of contrast prevented localization of discrete organelles.

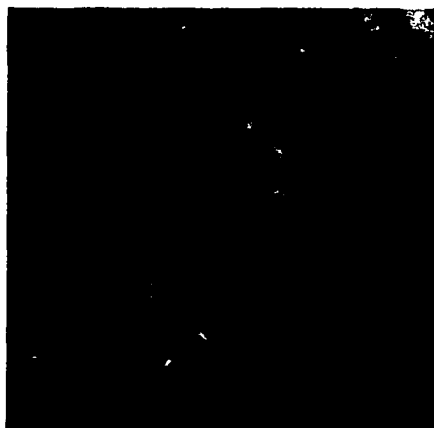
Phase observation of living cells revealed the presence of structures in the secretory cells, nearly at the resolution limit of the microscope, which underwent what appeared to be Brownian movement in the cytoplasm of these cells. Nearly all of the secretory cells burst during the course of one or two hours observation and, upon doing so, released the above particles. In this state, the speed of the par-

ticles was reduced and a dark ring could be seen to encircle each particle. It is doubtful that the rings were artifacts caused by movements of the particles between focal planes since orientation was provided by a single thickened region on the ring and the ring could be seen at all angles, like an equator, as the particles revolved and moved through the media. A second observation, at lower magnification, concerned movements within the tubule lumen as pressure was applied to the tubule. Masses of free cells or membrane-enclosed cytoplasm appeared to be packed inside the tubule and these droplets, inside of which the more refractive lipid droplets could be observed, could be made to slip past each other with slight pressure on the cover slip. If the distal tip of the tubule was broken with a needle, the droplets moved in both directions. Upon reaching the media outside the tubule, the droplets appeared to rupture, thus releasing their contents. Lipid droplets then rose to the surface of the media.

Electron microscopy

Cross sections through the blind tip of the tubule contained undifferentiated cells with large, indented nuclei and little cytoplasm (Fig. 17). The nuclei were smooth-surfaced and each contained a single nucleolus, sharply delimited by its high electron opacity. Developing endo-

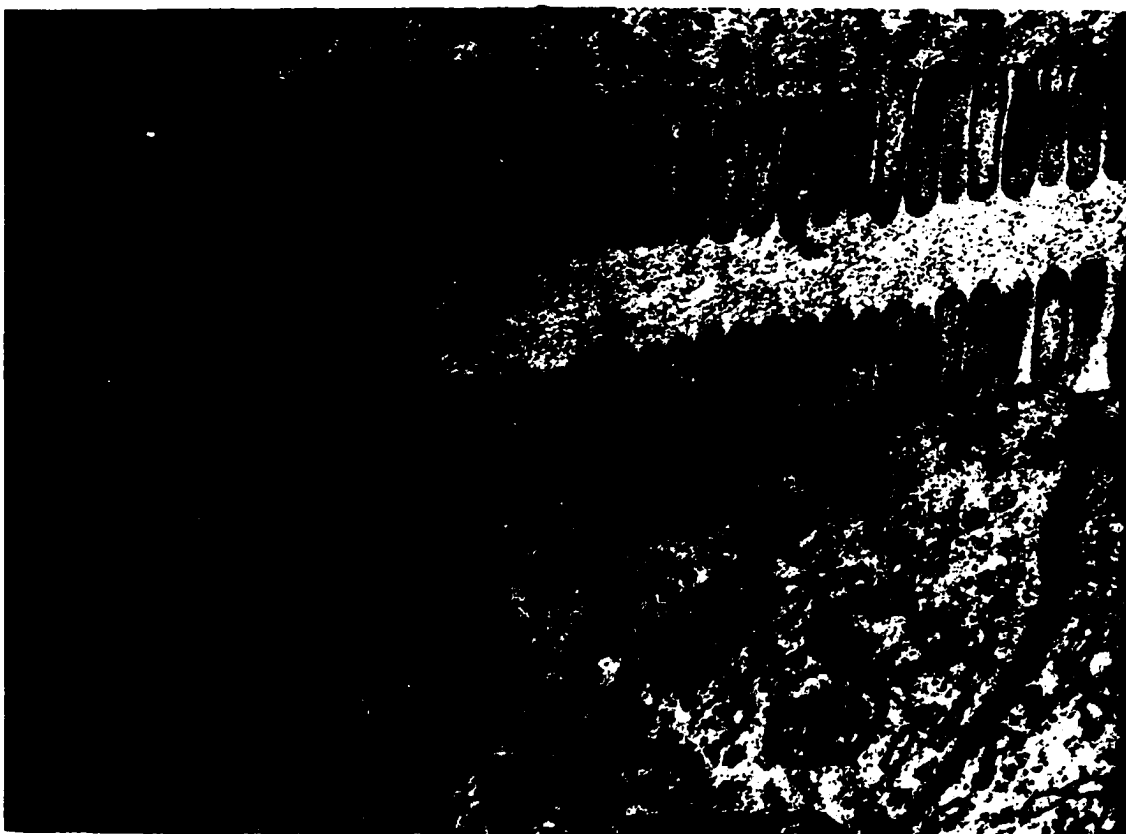
- Fig. 17. (top, left) Undifferentiated cells from blind tip
Electron micrograph. (x1910)
- Fig. 18. (top, right) Young absorption and fibrillar cells
Note continuity of striated border on both cell
types lining lumen (L). Electron micrograph.
(x3170)
- Fig. 19. (bottom) Young absorption and fibrillar cells
Fibrillar (F) and absorption (A) cells are
enclosed by a basement membrane (bm). A very
fine longitudinal muscle fiber (lm) lies in the
membrane. Electron micrograph. (x6450)



plasmic reticulum, both smooth and rough, appears in short lengths with either open or collapsed intracisternal spaces.

About 0.1 mm. from the tip of the tubule two other cell types appear (Figs. 18 and 19). One is a typical absorption cell with a brush border of blunt, closely packed microvilli, averaging about 0.6 micron long and 0.1 micron in diameter (Fig. 20), and an area nearly free of organelles, the terminal web, just below the microvilli. A layer of fine, particulate matter lies in the lumen on the surface of the brush border. Filamentous structures originating in each microvillus extend down into the apical cytoplasm for a distance of one micron or more. Mitochondria are concentrated just under the terminal web with many of their long axes orientated perpendicular to the cell's free surface. The cytoplasm contains large, irregularly shaped vacuoles (Fig. 18) which are electron transparent and probably contained fat prior to acetone dehydration. The most distinguishing characteristic of these cells is the heavy concentration of dense, asterisk-shaped granules, 18-36 millimicra in diameter (Fig. 21). (The cytoplasm of these cells gave a positive PAS reaction above.) Copper, and calcium granules seen by other investigators were not observed in this study. However, located near the junction of an absorption cell with its neighbor, are large, double-membrane enclosed areas (Fig. 22) whose cytoplasm is denser

- Fig. 20. (top) Absorption cell, apical portion
Striated border is made up of microvilli. Note
filamentous structures (arrows) extending from
each microvillus to cell interior. Electron
micrograph. (x33,700)
- Fig. 21. (bottom, left) Absorption cell, granules
Cytoplasm is rich in asterisk-shaped granules
(g) resembling liver glycogen. Electron micro-
graph. (x46,600)
- Fig. 22. (bottom, right) Absorption cell, inclusion
Double membrane-bound inclusion (i) contains a
denser cytoplasm than rest of cell and one or
more crystalloids (c). Electron micrograph.
(x5,480)

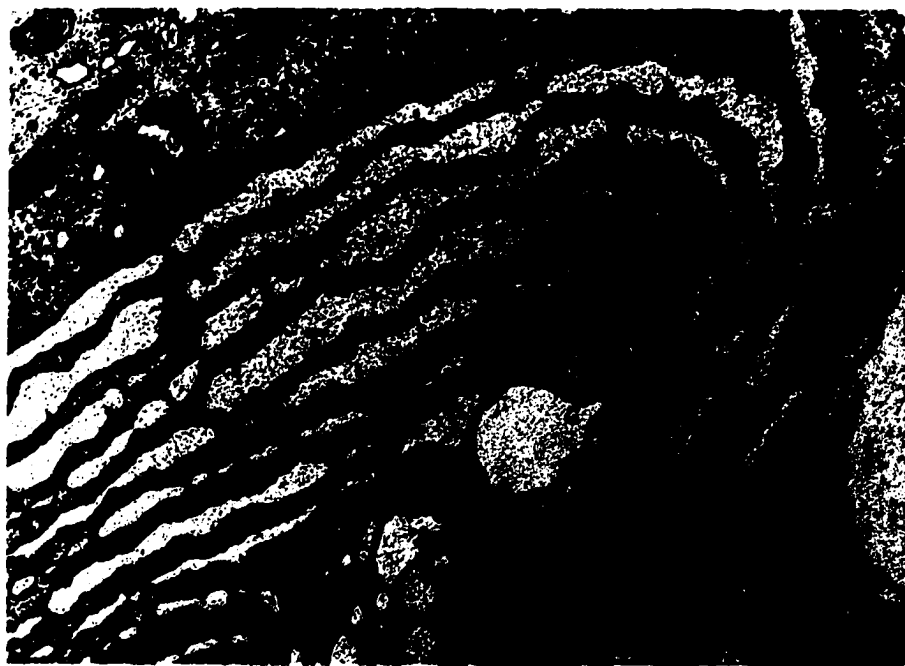
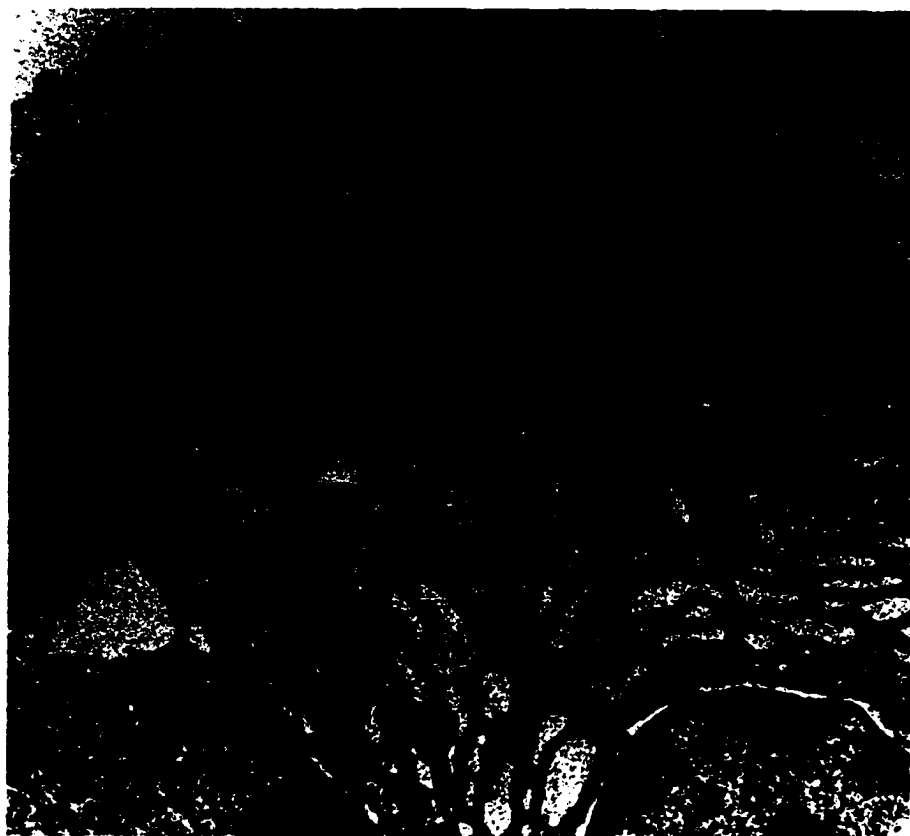


than that of the absorption cell itself. Within the enclosed area are irregular vacuoles, large rough granules similar in size and shape to those in the absorption cell proper but fewer in number, and large discoidal crystalloids with a dense core. Abundant in the absorption cells are lysosomes and smooth endoplasmic reticulum with amorphous material in the intracisternal spaces.

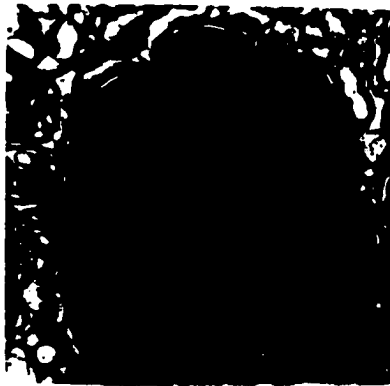
The second cell type in this region contains an abundance of rough endoplasmic reticulum with large intracisternal spaces and heavy concentrations of free ribosomes in the ground cytoplasm (Figs. 18, 19, 23 and 24). Near the blind end of the tubule the intracisternal spaces are round to oval and vacuolar in appearance (Fig. 23) while toward the middle of the tubule the spaces appear elongated, usually perpendicular to the cell surface (Fig. 24). These cells possess a brush border with microvilli of the same dimensions as that of the absorption cells but whose filamentous extensions into the apical cytoplasm are fewer and less distinct (Fig. 18). No terminal web is seen and the mitochondria are dispersed throughout the cytoplasm. A large number of Golgi apparati may occur in a single cell. In cells near the distal end of the tubule (Fig. 23) the Golgi apparatus occurs as a compact group of tubules with a few lateral vesicles; but, in cells of the middle portion of the tubule (Fig. 25), the Golgi region is characterized

Fig. 23. (top) Young fibrillar cell
Intracisternal spaces are rounded and Golgi
region (G) has few vesicles. Electron micro-
graph. (x10,160)

Fig. 24. (bottom) Mature fibrillar cell
Cell contains elongated intracisternal spaces
and highly vesiculated Golgi region (G).
Electron micrograph. (x11,910)



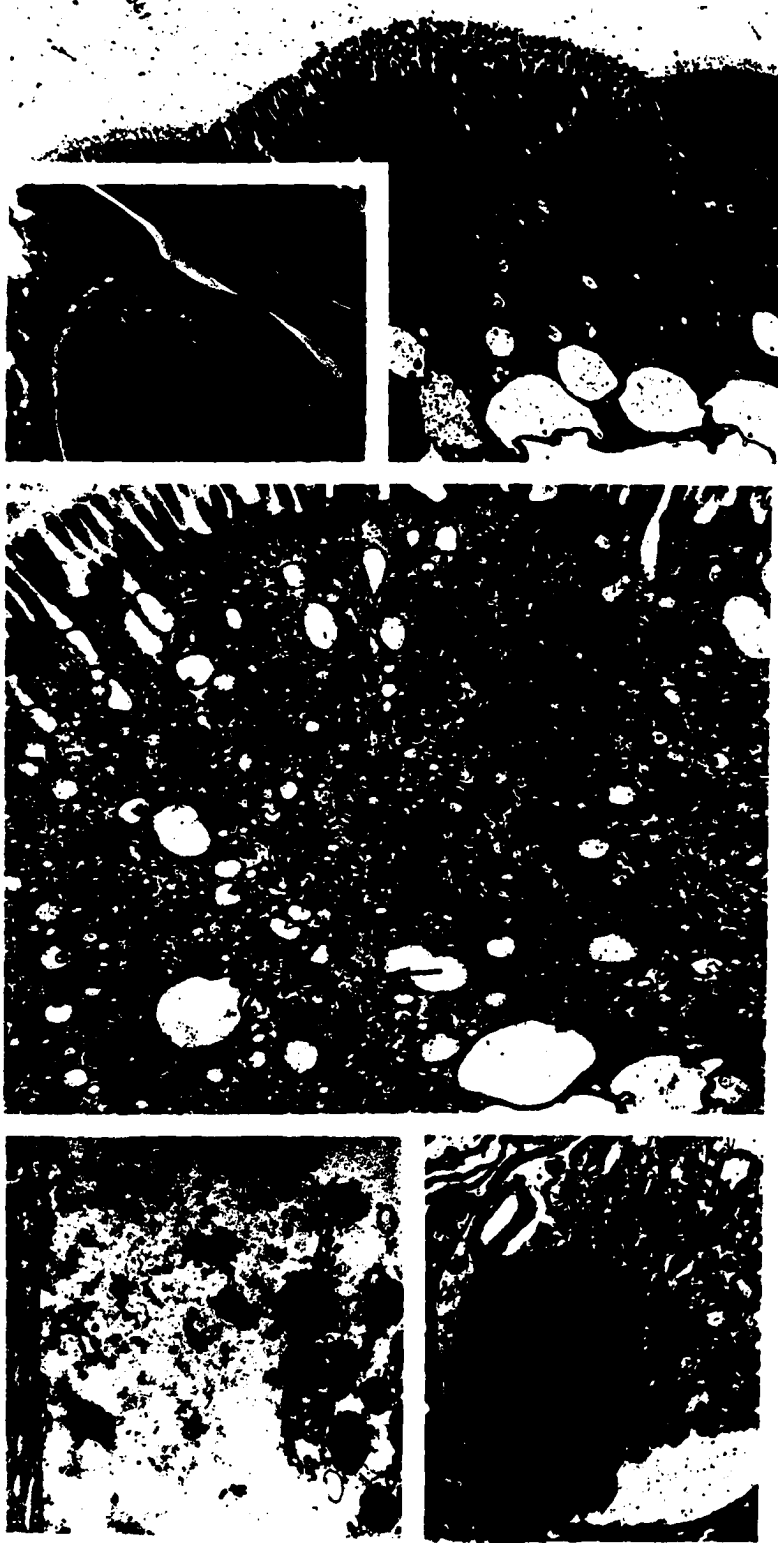
- Fig. 25. (top) Fibrillar cell
Many contacts are present between nucleus and cytoplasm (arrows). Golgi region (G) is vesiculated. Electron micrograph. (x14,000)
- Fig. 26. (bottom, left) Mature fibrillar cell
Nuclear surface is extremely wrinkled. Electron micrograph. (x9210)
- Fig. 27. (bottom, right) Fibrillar cell
Cell possesses a vacuole (V) filled with whorls of myelin figures and material resembling colloid. Electron micrograph. (x5610)



by a multitude of different sized vesicles with smaller ones in the center. Numerous multivesicular bodies and microtubules are also seen. Nuclei appear highly wrinkled in cells located in the middle of the tubule (Fig. 26) with suggestions of many contacts between cytoplasm and nucleoplasm (Figs. 25 and 26). (Nuclei in this region of the tubule showed an increased protein staining in the above cytochemical studies.) Occasionally a large vacuole is present (Fig. 27) and filled with numerous myelin figures characterized by whorls of membranes in a large, electron transparent vacuole. No evidence was found for the large, dense iron granules reported by other authors.

About 0.5 mm. from the blind end of the tubule, secretory cells (Fig. 28) make their appearance and continue for about 60% of the length of the tubule. The cells contain a single, large vacuole which is separated from the lumen by a narrow apical structure (Figs. 28 and 29) consisting of brush border, dense cytoplasm, pinocytotic vesicles of all sizes, mitochondria elongated toward the cell surface and bundles of microtubules (Fig. 30) also pointing toward the surface. The interior of the vacuole is, for the most part, electron transparent except for some scattered remnants of rough ER and a few large secretory granules (Fig. 31). (The latter gave positive results with cytochemical tests for protein and polysaccharides in the

- Fig. 28. (top, inset) Secretory cell
Phase micrograph of thick section prepared for electron microscopy. Note definite apical structure. (x2,190)
- Fig. 29. (top) Secretory cell, apical structure
Active pinocytosis is present in this region. Mitochondria are elongated and oriented toward apical surface. Electron micrograph. (x5,000)
- Fig. 30. (center) Secretory cell, apical structure
Bundles of microtubules (arrows) are also oriented toward luminal surface. Closer examination revealed that entire apical structure was filled with microtubules. Electron micrograph. (x21,200)
- Fig. 31. (bottom, left) Secretory cell, vacuole
Secretory vacuole contains mainly amorphous material with scattered remnants of rough endoplasmic reticulum and some secretory granules. Electron micrograph. (x10,760)
- Fig. 32. (bottom, right) Secretory cell, vacuole
Occasionally, a vacuole of myelin figures is present similar to that in the fibrillar cell shown in Fig. 27. Electron micrograph. (x6,300)



above section.) Occasionally, the apical structure appears to be torn away from the cell on one side. In such cells, the cytoplasm and organelles surrounding the secretory vacuole are in a state of degeneration. Occasionally present is a large vacuole filled with myelin figures (Fig. 32) and similar to that seen in fibrillar cells.

Investing the entire tubule is a fibrous basement membrane averaging 0.5 micron thick (Fig. 33). Located in the membrane is a network of muscle fibers (Figs. 16, 19, 34, 35) consisting of (a) regularly spaced circular fibers, about 0.75 micron in diameter, and (b) fine longitudinal fibers about 0.2 micron in diameter, which appear to connect the circular fibers. Longitudinal sections through these fibers show no evidence of striations; however, cross sections show large and small filaments within the fibers arranged in a geometric pattern.

Discussion

Basic cell types in the hepatopancreas tubule

The four cell types observed in this study and their locations in the hepatopancreas tubule correspond to those previously described in the literature despite differences in nomenclature.

Undifferentiated cells These are small cells at the distal tip of the tubule possessing a relatively large nu-

Fig. 33. (top) Basement membrane (bm)
Electron micrograph. (x13,710)

Fig. 34. (center) Muscle fibers
Oblique section through two muscle fibers shows
geometric pattern of myofilaments within fibers.
Electron micrograph. (x32,200)

Fig. 35. (bottom) Muscle fibers
Unusual section shows both longitudinal muscle
fiber (lm) cut lengthwise and circular muscle
fiber (cm) in cross section. Electron micro-
graph. (x15,400)



cleus, developing endoplasmic reticulum (both smooth and rough) and other organelles typical of undifferentiated cells. They correspond to the E-cells of Jacobs (26), the embryonic cells of Davis and Burnett (6), the mother cells of Frenzel (15) and the undifferentiated cells of Ogura (43).

Absorption cells Appearing just proximal to the blind end of the tubule, these cells contain large lipid deposits and their cytoplasm is rich in polysaccharide granules. Similar granules are seen in mammalian liver cells and are usually identified by investigators, e.g. Rhodin (48), as glycogen granules. The striated border is strikingly geometric and composed of blunt, even microvilli. From the border, filamentous extensions pass through a terminal web into the deeper cytoplasm. Thus, these cells are similar in fine structure to absorption cells in the mammalian intestine (48). Light microscopy reveals the presence of green, irregularly shaped crystals which may be copper granules. E.M. sections revealed large, double membrane-enclosed inclusions resembling the copper granules of Miyawaki and Tanoue (42) in Procambarus. These cells correspond to the fat cells of Frenzel (15), the hepatic cells of Dorman (7), the R-cells of Jacobs (26), the light cells of van Weel (54), the Cu-cells of Ogura (43) and the absorption cells of Davis and Burnett (6).

Fibrillar cells This term refers to the fibrillar appearance of the cells' cytoplasm under light microscopy. However, the appearance is not due to artifact but to the coarse and elongated rough endoplasmic reticulum and swollen intracisternal spaces in these cells, particularly in mature fibrillar cells of Region II. A similar ER system was observed by Ekholm (9) in mouse thyroid follicle cells which elaborate colloid.

The intense basophilia seen in light microscopic preparations is a result of the high ribosome concentration, both membrane-bound and free, in these cells. Yamamoto (59) in 1960 reported the degeneration of these cells after eyestalk ablation and stated this could be prevented by implanting eyestalk extracts. Miyawaki et al. (40) later demonstrated that the basophilia of the fibrillar cells was due to RNA and Miyawaki and Tanoue (42) reported degeneration of the endoplasmic reticulum after eyestalk ablation. Fingerman et al. (11) reported a decrease in the RNA content of the hepatopancreas after eyestalk ablation using both histochemical techniques to study the Fe-cells and spectrophotometric assays of RNA extracts from gland homogenates. The decrease was inhibited by injections of eyestalk extracts. In 1965 Fingerman and Yamamoto (12) correlated an increased pH and decreased amylase activity in digestive juice with this loss of RNA.

The cytoplasm of these cells also shows a high protein content but it is difficult to determine the exact reason. The protein may be in the cytoplasm, the intracisternal spaces or the ribosomes or it may simply be an artifact due to the packed membranes in the cell.

Irregularities in the nuclear surface and the several points of contact between nucleus and cytoplasm suggest some interchange of material between these two regions. Kurosumi (32) mentions that certain types of secretory cells have characteristic, irregularly-surfaced nuclei, especially those cells involved with mucous or mucoid secretion. He suggests that this type of nuclear surface makes possible an active nucleocytoplasmic interaction by providing much greater surface areas on the nuclei.

The role of the fibrillar cell, as evidenced by the elaborate ER system, the ribosome content, the vesiculation of the Golgi, and the swollen intracisternal spaces, is obviously synthetic. However, the material it synthesizes has not been established and this problem is related to the sequence of cell differentiation in the tubule. The large vacuole seen in some cells and filled with whorls of myelin figures and spiral lamellae is similar to the system of membranes described by Schulz and de Paola (50) which they called the delta-cytomembranes and which, according to Kurosumi (32), have since been observed in a variety of cell

types. Schulz and de Paola postulated that the structure has a role in the synthesis of mucopolysaccharides and possibly in mucous secretion. Miyawaki et al. (40) reported that the Fe-containing vacuole was the only region in these cells rich in mucopolysaccharides or mucoprotein bound with iron. It appears therefore, that the fibrillar cells are capable of synthesizing an iron-associated polysaccharide or mucoprotein. However, until the exact nature and role of the iron-polysaccharide is elucidated, these observations remain a mystery. The iron may be a waste product, perhaps accumulated from the degradation of porphyrin groups from digested tissues, or it may serve some metabolic purpose for the crayfish, for example, as an enzyme activator in the same manner it activates peptidases in certain microorganisms (16). In regard to the latter function, it is interesting to note that the powerful trypsin inhibitor present in egg white is a mucoprotein (16).

In addition, there appears to be a relationship between the calcium- and iron-storing functions of this cell. Yonge (60) and van Weel (54) observed active iron absorption by fibrillar cells and Ogura (43) found that eyestalk ablation, which is a molt-inducing operation, stimulates Fe-granule discharge. Miyawaki and Sasaki (41) reported that calcium is actively taken up after removal of the eyestalks and Miyawaki et al. (40) observed that the hepatopancreas is

richer in this metal during molting presumably because the gland is storing calcium to be used in forming the new exoskeleton. Thus, the same stimulus which causes a general decrease in normal metabolic functions and RNA concentration and, in general, prepares the animal for molting, simultaneously effects iron discharge and calcium resorption.

Fibrillar cells correspond to the F-cells of Jacobs (26), the dark cells of van Weel (54), the Fe-cells of Ogura (43) and the fibrillar cells of Davis and Burnett (6). Possibly, they are also the same as Frenzel's enzyme-replacement cells (15).

Secretory cells These cells do not resemble mammalian pancreatic, liver or gastric secretory cells. Because of its large secretory vacuole with protein and PAS-positive granules, the hepatopancreatic secretory cell, to some extent, resembles the mucous cell of the sublingual gland described by Kurtz (33). The similarity is also seen when the fine structure of these two cells are compared. In both, there is a single large vacuole which is mainly electron transparent except for an assortment of granules and remnants of rough ER. Also, the lateral basal portions of both cells are filled with a very dense cytoplasm and endoplasmic reticulum which seem to be in a state of degeneration. The similarity, however, ends here because the hepatopancreatic secretory cell possesses an apical struc-

ture unlike that of the mucous cell. The apical complex of the former is PAS-positive, has a striated border similar to the absorption and fibrillar cells, and shows evidence of very active pinocytosis. The apical portion of the sublingual gland mucous cell does not possess a striated border and is free of visible pinocytotic activity. In regard to its apical portion, the secretory cell of the hepatopancreas resembles the intestinal goblet cell since the latter possesses a striated border. Palay (44) has studied the fine structure of the latter and, interestingly, the "resting" goblet cell does not look like the textbook descriptions of a goblet cell and can only be distinguished from adjacent absorption cells by the density of its cytoplasm, a situation not unlike that of the hepatopancreatic secretory cell. Secretion in the goblet cell is both holocrine and apocrine; that is, as secretory material is synthesized it begins to fill the cell in the form of vesicles which gradually enlarge and coalesce into a single vacuole. Extrusion occurs when the cell membrane ruptures and material is released freely into the exterior rather than pinched off within a membrane as in typical apocrine secretion. Nevertheless, the basal portion of the cell remains intact, with the nucleus, and forms another resting cell. The latter, of course, does not occur in classical holocrine secretion. The results of this study, using

osmium impregnation, showed the presence of apocrine secretion after feeding. Travis (53) similarly observed apocrine secretion in Homarus.

Another interesting observation by Palay (44) was that the increased density of the goblet cell cytoplasm is due to the presence of "densely matted, fine fibrils" especially in the apical cytoplasm. In the present study, masses of oriented microtubules were found in the apical structure directed toward the striated border. Their presence is interesting in view of current concepts (1) associating several diverse forms of cell movement, including mitosis, muscle contraction and cytoplasmic flow, with the presence of filaments and microtubules. There is no evidence, however, to show that the microtubules in the secretory cell are not passively oriented by the obviously active flow of materials across the striated border. Conversely, the microtubules may be involved in the extrusion phase of the secretory process. Similar structures are present in electron micrographs of the secretory cell in snail hepatopancreas in an article by David and Gotze (5).

The secretory cells correspond to the enzyme cells of Frenzel (15), the pancreatic cells of Dorman (7), the B-cells of Jacobs (26), the extrusion cells of van Weel (54), and the secretory cells of Davis and Burnett (6).

Mode of secretion in the hepatopancreas

Confusion in the nomenclature of the various cell types resulted from the fact that some investigators chose names on the basis of morphology and others according to supposed function and each system contained many opposing views. However, most of the differences can first be grouped into one of two theories regarding mode of secretion.

The theories proposed by Hirsch and Jacobs (21, 22) and by van Weel (54) on the mode of digestive enzyme secretion in the hepatopancreas differ in that the former is based on linear secretory cell differentiation followed by holocrine secretion while the latter depends on cyclic differentiation and merocrine secretion. The difference might be resolved simply by assuming that the mode of secretion in crayfish is different from that in crabs since these were the two animals used. This assumption is not entirely warranted, however, for many reasons. (1) The anatomy and morphology of the hepatopancreas in the two animals is quite similar; (2) also similar are the basic cell types in the tubule and their locations and arrangements into three zones in the epithelial layer; (3) the two studies utilized the same technique of stimulation by starvation-feeding and then counting cells and plotting their numbers against time after feeding; (4) both theories propose that all cells in the tubule epithelium, including

the secretory cells, have their origin in the embryonic cells at the distal end of the tubule; and (5) the basophilic, fibrillar cell (or dark cell) is an intermediate stage in secretory cell formation in both theories. Aside from terminology, therefore, the main difference is that Hirsch and Jacobs postulate that secretion is an endpoint, that the cells are lost and must be replaced via another wave of linear cell differentiation while van Weel concluded that, in secretion, the secretory cell is not lost but is reformed and more secretory material is synthesized in cyclic restitution.

In spite of the similarities described above, there is one important difference in the procedures employed. Hirsch and Jacobs made observations every half-hour for $6\frac{1}{2}$ hours after feeding while van Weel studied the gland for a total of 36 hours after feeding, with time intervals between observations ranging from an initial 30 minutes up to six hours at the end of the period. Thus, in spite of the apparent conflict between the conclusions of these two studies, a comparison is almost impossible because of differences in the time scales. Hirsch and Jacob's work gives a precise picture of events during a relatively short period following feeding while van Weel's work presents an over-all picture of gross changes occurring over a very long period of time after feeding. Coincidentally, 36 hours is the approximate length

of time that food is retained in the crayfish alimentary tract. Therefore, the two studies complement rather than contradict each other and, since their conclusions are not mutually exclusive, they might be reconciled by stating that, in crustaceans, the immediate response to starvation-feeding is holocrine secretion but afterwards secretion is merocrine.

There are several factors which support this hypothesis: (1) Although Hirsch and Jacobs' data show good correlation among the cycling of numbers of various cell types, of mitotic indices and of enzyme activities, there is little agreement between the absolute number of mitoses and that of the various cell types. In all cases, the peak number of cell divisions was far lower than the B-cell maxima which, according to these authors' theory, resulted from it. The autoradiography studies of Davis and Burnett (6) led them to conclude that the mitotic cycle in the crayfish hepatopancreas is 3 to 5 hours in length. This value is about the same as that derived from Hirsch and Jacobs' studies but, more importantly, the rate is too slow to account for the discrepancy between cell number and number of cell divisions by assuming that the low mitotic frequency is overcome by a very short mitotic cycle. Therefore, since each B-cell is not derived from a separate mitotic division, some cyclic restitution of B-cells must take place which

reveals the presence of either merocrine or apocrine secretion.

(2) In Davis and Burnett's study, the H^3 -thymidine label appeared in the nuclei of embryonic cells within 15 minutes after injection into the crayfish circulation but was not observed in the nuclei of secretory cells until 24 hours. That is, the label appeared sometime between the observation at 10 hours and the next one at 24 hours. Hirsch and Jacobs found that approximately three hours were required for the differentiation of embryonic cells to secretory cells. Since the autoradiography study did not utilize a starvation-feeding stimulus but was conducted using non-starved animals, we can assume that its results more closely reflect the situation in the normal animal and that between 10 and 24 hours are required to replace lost secretory cells from embryonic cells. From Hirsch and Jacobs' work it would appear that starvation-feeding results in the loss of vary large numbers of secretory cells and that, immediately after such stimulation, the replacement process is accelerated and requires only about 3 hours to complete. The picture is made more complete by van Weel's results showing peak numbers of secretory cells at 1, 12 and 24-27 hours after starvation-feeding, indicating that restitution requires 11-13 hours when an over-all view of both immediate and long-range effects is examined. This

figure is in agreement with that obtained by Davis and Burnett.

(3) In all of these studies, the physiology of the hepatopancreas has been largely ignored above the level of the cell. The present study has confirmed the presence of a system of muscle fibers in the basement membrane of the tubule and this system is capable of contracting the tubule and changing its shape from a long, slender tube with a relatively smooth surface to a shortened series of bulbous expansions separated by sphincter-like constrictions but having a common lumen and resembling, in miniature, a segment of mammalian intestine in extreme peristalsis. The physiological control of this system has not been investigated but, from observations of contracted tubules under the dissecting microscope, one can easily assume that the contractions exert great pressures both within the tubule lumen and also within the cells. It is conceivable that such pressures, in addition to their effecting a flow of material within the lumen, might also play a role in aiding the flow of material either into or out of the cells depending on the relative pressures on the two sides of the cell membrane. Of even greater probability is the possible role of these pressures in either rupturing the secretory cell membrane or squeezing out whole cells as in classical holocrine secretion. If the assumption is made that long

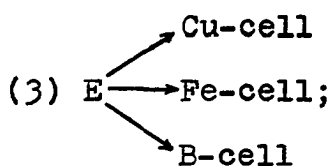
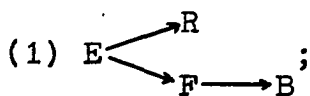
starvation followed by feeding is an extreme stimulus and that the tubule contractions which it produces are of greater strength than normal, then the logical conclusion is that starvation-feeding will result in a greater number of secretory cells undergoing holocrine secretion than in the animal which has been fed without prior starvation. In the latter, the weaker contractions would result in a lower incidence of holocrine secretion. Since Hirsch and Jacobs used starvation-feeding, this may explain why they found three-hour replacement periods while Davis and Burnett, using non-starved animals, found a 10-24 hour period necessary to replace secretory cells. van Weel also used starvation-feeding as a stimulus but his time intervals increased almost geometrically from 30 minutes to six hours so that only a general picture of the cellular changes could be provided. In spite of this, van Weel's findings show both an initial peak at one hour and a second peak 12 hours later thus reflecting the immediate effects (those studied by Hirsch and Jacobs) and the long term effects (studied by Davis and Burnett). That is, the one hour peak may be the same as the first peak of Hirsch and Jacobs and due to holocrine secretion while the 12 hour peak (measured long after the violent reaction to the starvation-feeding stimulus subsided) represents the resumption of normal secretory mechanisms, i.e. merocrine secretion, to continue

digestion of nutrients in the alimentary tract.

From these observations, the original point of conflict regarding mode of secretion, viz. holocrine vs. merocrine, may be resolved by assuming that both types may be present. A strong stimulus such as starvation-feeding, might result in massive holocrine secretion within an hour or so, due possibly to extreme contractions of the muscle network surrounding the hepatopancreas tubule (although extreme stimulation may also act on the cells themselves as in pilocarpine treatment). If this were the case, then the following speculation is suggested: In the normal state when feeding is regular and not interrupted by long periods of fasting, secretion is not holocrine but merocrine or apocrine and secretory cell replacement is mainly via cyclic restitution.

Sequence of cell differentiation

It will be recalled that four different theories have been postulated concerning the sequence of cell differentiation in the hepatopancreas. These are:



and (4) $E \longrightarrow R \longrightarrow B \longrightarrow F$; proposed, respectively, by Hirsch and Jacobs (22), van Weel (54), Ogura (43) and Davis and Burnett (6). Once again, it should be emphasized that all of these may be correct since different species of crayfish and one crab were used while, at the same time, similarities in the anatomy and cytology of the hepatopancreas of the four animals utilized is striking. Nevertheless, the question should be asked whether it is feasible that actually a single system of differentiation exists here which when studied by different methods presents the characteristics of a variety of systems.

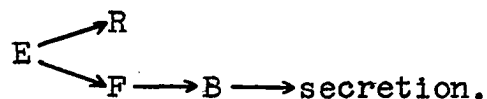
Separate origins of F-cell and R-cell From the present study, where serial sections through an entire tubule were examined, undifferentiated cells were restricted to the distal tip of the tubule, absorption and fibrillar cells both began to appear about 0.25 mm. from the tip of the tubule, secretory cells occurred in the middle 50% of the tubule, and the most proximal portion of the tubule contained a few pycnotic secretory cells but mainly absorption and fibrillar cells. The most obvious conclusion from this localization of cell types is that absorption and fibrillar cells either have separate origins or one is capable of making a very rapid transformation into the other while migrating only a few micra in the tubule. The latter alternative is unlikely for the following reasons: (1) Both

light and electron microscopy reveal vast morphological differences between the two cell types including the presence of large glycogen and lipid deposits, "green crystals", rich concentrations of ribosomes and an elaborate rough endoplasmic reticulum to name some of the differences. In fact, the only similarity is the presence of a striated border in both cells. (2) Both types undergo maturation along the length of the tubule. In the absorption cell the lipid droplets become altered in size and location while in the fibrillar cell there is an elongation and orientation of the ER and a vesiculation of the Golgi apparatus. From these observations it may be concluded that absorption and fibrillar cells have separate origins as proposed by Hirsch and Jacobs (22) and Ogura (43).

Differentiation of F-cells into B-cells It was also observed in the serial sections of this study that fibrillar cells appear prior to secretory cells in the tubule, that is, closer to the region of cell division in the distal tip. This indicates that fibrillar cells are correctly positioned for differentiation into secretory cells but that the reverse could not occur unless cyclic restitution takes place. That is, the same picture would result if secretory cells were first differentiated from fibrillar cells and, after extrusion, appear again as fibrillar cells to carry on the cycle.

Before examining this last point, the main question must be resolved: Do either absorption or fibrillar cells give rise to secretory cells or do the latter arise independently? Hirsch and Jacobs, among others, have shown that a transition of the basophilic cells occurs along the tubule. This transition includes a gradual size increase and coalescence of their vacuoles terminating in the typical secretory vacuole of the B-cell. This was taken as strong evidence for differentiation of F-cells into B-cells. The present study showed other similarities in morphology between fibrillar and secretory cells including a high ribosome concentration and the presence of delta-cytomembranes whereas several differences exist between absorption and secretory cells which need not be reiterated here. Thus, on the basis of the prior appearance of F-cells in the tubule and the similarities between F-cells and B-cells, it is concluded that F-cells differentiate into B-cells.

Fate of the secretory cell In view of the above conclusions, the following scheme may be presented showing how, at least initially, the various cells types are differentiated:



This, of course, is the scheme of Hirsch and Jacobs (22). However, the latter investigators postulated that only

holocrine secretion took place in the hepatopancreas. In the present study there are several indications that holocrine secretion is not the major extrusion mechanism in this gland. The Azure-A triple stain showed PAS-positive material being extruded from a secretory cell in the classical merocrine fashion and the osmium-impregnation technique revealed the presence of apocrine secretion after feeding. Electron microscopy of the secretory cell revealed very active pinocytosis in the apical structure which might indicate that some form of merocrine secretion is utilized, possibly what Kurosumi (32) refers to as eccrine secretion: Either merocrine or apocrine secretion implies cyclic restitution and, if this is present in the hepatopancreas tubule, there should be some evidence for van Weel's empty cells and dark cells. In the present study, the middle region of the tubule, although predominately a secretory zone, possessed both absorption and fibrillar cells interspersed among the secretory cells. Thus, either of these could be the "post-secretory" cell. However, as described above, there are great morphological similarities between fibrillar and secretory cells while absorption and secretory cells have little in common. Thus, if cyclic restitution is present, the most logical intermediate form of the secretory cell is the fibrillar cell and cycling would include the repeated transformations of fibrillar cells

into secretory cells and, after extrusion, back again into fibrillar cells. This scheme is similar to that proposed by van Weel in which cyclic restitution involved a dark cell precursor of the secretory cell. van Weel's light cell, which he placed between the dark cell and the extrusion cell, cannot be accounted for unless one supposes either a mistaken inclusion of the absorption cell into the scheme or that the light cell is a young secretory cell (e.g. the B₁ and B₂ cells of Hirsch and Jacobs) neither of which is a satisfactory explanation in view of van Weel's careful and detailed cytological descriptions.

Davis and Burnett's autoradiographic studies offer some obstacles to the above scheme. Their results showed that the label appeared in embryonic cells in 10-15 minutes, absorptive cells in $\frac{1}{2}$ -2 hours, secretory cells in 10-24 hours, and fibrillar cells after 1-2 days; i.e. these times represent two successive observations with the label absent in the first and present in the second. Since secretory cells picked up the label at least 24 hours prior to the fibrillar cells, this was interpreted by the authors to mean that secretory cells differentiate into rather than from fibrillar cells. This absence of labelling in the fibrillar cells prior to secretory cells appears to conflict with the above scheme that secretory cells originate from fibrillar cells even if the presence of cyclic restitution is accepted.

However, they do not disprove the scheme if one carefully examines the time scale upon which observations were made. In their experiments, Davis and Burnett have several long periods of time during which no observations were made and this point is brought out in their Discussion (6). Most relevant to the present discussion is the 14-hour period between 10 and 24 hours after injection of the radioactive sample. The possibility must be considered whether, during this 14-hour period, fibrillar cells could have been formed from embryonic cells and then differentiated into secretory cells thus giving the impression that fibrillar cells were never present en masse during this period. A corollary question is why fibrillar cells are not labeled until 1-2 days post injection.

It is impossible to answer these problems fully on the basis of available information, either from the literature or this study, since too many unknowns are involved. However, some speculations can be made. Hirsch and Jacobs concluded from their studies that the transformation from E-cell to F-cell took 2 hours and from F-cell to B-cell, 1 hour. Since we have assumed that these times are accurate only after extreme stimulation, the absolute values cannot be applied to Davis and Burnett's work. However, the metabolic processes effecting these differentiations are probably the same in both cases with only a change in rate. If

it is assumed that the ratios of the required times are the same, no matter what type of stimulus is applied or what the absolute times are, then it requires only half the time to convert F-cells to B-cells as it does E-cells to F-cells. Davis and Burnett's results show that label appears in the secretory cells 10-24 hours after injection thus indicating that differentiation of secretory cells from embryonic cells in non-starved crayfish takes between 10 and 24 hours. Using the 2:1 ratio from Hirsch and Jacobs' work and the maximum time, 24 hours, for complete secretory cell formation, it would require 16 hours to convert E-cells to F-cells and 8 hours to form F-cells into B-cells. The crucial time, 16 hours, thus falls into the 10 to 24-hour period during which Davis and Burnett made no autoradiographic observations. In fact, using these criteria, the time required for complete differentiation of secretory cells from embryonic cells could have varied anywhere from a little more than 15 to 24 hours without producing a label in the fibrillar cells at either 10 hours or 24 hours post-injection. And, from such results, one would receive the impression that secretory cell formation does not occur via fibrillar cells.

However, label did appear in the fibrillar cells after 1-2 days. This observation does not conflict with the suggested scheme of differentiation since the latter included

reformation of fibrillar cells after extrusion of secretory material as part of the cyclic restitution process. In regard to timing, much of the synchrony of differentiation, which began with the simultaneous uptake of tritiated thymidine by several undifferentiated cells in the blind tip, would be expected to disappear after one or two days especially if cyclic secretion were in operation. Thus, the time of appearance of label in the fibrillar cells is not pertinent to the proposed scheme of differentiation nor is there any available evidence which might confirm or contradict this time.

Admittedly, the above speculation does not prove which is the correct sequence of differentiation; however, it does show that the data of Hirsch and Jacobs and that of Davis and Burnett, although opposite in their respective conclusions, do not really conflict when placed on the same time scale before comparison. Because of differences in method and timing, a critical point such as F-cell formation in one study has no means of confirmation or disproof in the other.

Evidence against independent B-cell formation

Ogura's suggestion that Cu-cells, Fe-cells and B-cells each arise independently of the others from E-cells is based on several factors including the spatial locations of the cells in the tubules. But the crucial factor was the asso-

ciation of fibrillar cells with iron granules, absorption cells with copper granules, and the absence of these metals in secretory cells. Ogura was able to follow the maturation of metal granules (or their precursors) and secretory vesicles in their respective cell types along the length of the tubule and used these inclusions as a marker in specific cell types. In the case of Fe-cells, although iron-granules themselves did not appear until Region II, minute, ferric-positive particles were observed in cells of Region I.

Ogura concluded that these particles were precursors of iron granules. Because he was able to observe the markers as far back as the region of embryonic and transitional cells, he concluded that the three cell types had separate origins beginning at the blind end of the tubule which meant the existence of three separate lines of differentiation for Cu-cells, Fe-cells and B-cells.

Since the present study and that of Hirsch and Jacobs indicates independent formation of absorption and fibrillar cells, the only conflict concerns B-cell formation. Ogura indicates in his Discussion (43) that it "may not reasonably be supposed that either iron or copper is so readily replaced by the other in the same granule or so suddenly lost in situ without the granule being lost from the cell;" that is, the hypothesis that the three cell types arise independently is itself dependent on the stability of

the metal granules for if, as an example, iron granules were discharged from Fe-cells, the latter would then be difficult to distinguish from a young secretory cell thus leaving open the possibility that Fe-cells are precursors of B-cells and conflict with the concept of independent differentiation. In the same paper, however, Ogura reports that the metal-containing cells undergo changes in various conditions of the animal. During starvation, there is a decrease in ferric-positive granules in the Fe-cells; during breeding, Fe- and Cu-granules are found in the feces indicating discharge by the cells; and finally, eyestalk extirpation causes a marked enlargement of both Fe- and Cu-containing vacuoles in their respective cells and an increased excretion of these metals in the feces. Miyawaki et al. (40, 42) have studied the cytochemistry and electron microscopy of the metal-containing cells and found that a correlation exists between the metal concentration in the hepatopancreas and its concentration in the animal's external aquatic environment. In the present study, only structures resembling other authors' descriptions of Cu-granules were observed in both light and electron microscopy preparations; no evidence was seen for the Fe-granules. It would seem from all of these observations that Fe-granules are not as stable or as universally present as the Cu-granules nor do the former seem to be essential for normal functioning of the hepato-

pancreas. Miyawaki suggests that, at least for the Cu-granule, if the metal is in too high a concentration in the crayfish's environment, it simply becomes concentrated in hepatopancreas as a detoxification measure. He also presents some evidence for Fe-granule discharge. In addition, van Weel (54) fed iron-saccharate to crayfish and found that practically all cells in the tubule had ferric-positive particles in their cytoplasm within a short period of time. Thus, it would appear that neither Fe- nor Cu-granules have the stability required to serve as a cell marker in following differentiation. The point is academic in the case of Cu-granules since the absorption cells also possess the more characteristic lipid and glycogen deposits. However, since only the possession of an iron granule or rather, ferric-positive particles assumed to be precursors of iron granules, conflicts with a mass of evidence linking fibrillar cells and secretory cells, and since the presence of the iron is subject to a variety of hormonal and environmental conditions, the probability of independent formation of secretory cells seems far less likely than their differentiation from fibrillar cells.

Thus, from a critical examination of supposedly conflicting theories, and a comparison of previous investigations with the present study, it would appear that cell differentiation in the crayfish hepatopancreas is neither

exclusively linear nor cyclic but a combination of the two. The various cell types in the tubule epithelium are formed, initially, in the manner described by Hirsch and Jacobs but secretory cells may be reconstituted while passing through a fibrillar cell stage. In addition, absorption cells are apparently formed independently from the fibrillar-secretory cell line. In all probability, the main path of differentiation is cyclic, the linear portion being used to produce replacement cells.

Conclusions

1. The hepatopancreas of O. virilis has the same anatomy, basic cell types and tubule regions as that of previously studied crayfish and the crab Atya spinipes.
2. Electron microscopic observations of the basic cell types revealed that the PAS-positive reaction in the cytoplasm of absorption cells is due to the presence of large masses of granules identical in size and form to the glycogen granules in liver cells; that the basophilia of the fibrillar cell is due to rich concentrations of free and membrane-bound ribosomes and the light microscopic fibrillar appearance of this cell originates from a coarse system of rough ER and swollen intracisternal spaces orientated in an apicobasal direction; and, that secretory cells possess a complex apical

structure which actively carries on pinocytosis and includes orientated mitochondria and microtubules.

3. Secretion by the hepatopancreas secretory cells in the normal crayfish is primarily either merocrine (eccrine) and/or apocrine with no evidence of holocrine secretion.
4. Extreme stimuli, such as prolonged starvation followed by feeding, may initially produce massive holocrine secretion which is probably due to intense tubule contractions. As the cell population is gradually returned to normal levels, there is resumption of primarily merocrine or apocrine mechanisms.
5. Secretory cells are differentiated from embryonic cells in the distal tip of the tubule via fibrillar cells. Absorption cells are differentiated independently. These pathways are used in replacing cells lost either in small numbers, due to age and wear, or in massive quantities, due to abnormally stimulated holocrine secretion.
6. Merocrine secretion is followed by restitution of the secretory material and the secretory cell via the fibrillar cell stage.
7. Previous, conflicting theories regarding mode of secretion and sequence of cell differentiation in the hepatopancreas tubule can be explained by the fact that

no two investigators used the same methods and time scale and, thus, were probably observing different facets of the same process. Analysis of these investigations and comparison with the present study shows that the apparently conflicting results were based, in most cases, on complementary and even confirming data.

PART II. RHYTHMIC CHANGES IN PROTEASE
ACTIVITY AFTER FEEDING

Introduction

Cytological observations made in Part I were necessarily restricted by the static nature of fixed tissue sections; therefore, any conclusions regarding dynamic processes acting within the hepatopancreas must be considered more or less speculative in the absence of non-histological confirmation. In Part II, such confirmation is sought from studying fluctuations of enzyme activity and protein content after feeding.

The basis for these studies can best be understood in terms of Hirsch and Jacobs' work (21, 22). These investigators found rhythmic changes of several histological parameters in the crayfish hepatopancreas tubule after starvation-feeding. When these fluctuations were placed on a single time scale, an ordered and reproducible sequence of maximal values of the parameters was observed. According to the authors, this sequence enabled them to follow the complete differentiation of secretory cells, from their origin in mitoses at the distal tip of the tubule to their maturation and extrusion of secretory products in Region II of the tubule. The most important conclusions from these studies, in relation to the experiments in this section were

that (1) secretion was exclusively holocrine and, (2) the rate-limiting factor of rhythmic secretion, as well as the source of all the above rhythmicities, was the cycling of mitotic divisions in the blind end of the tubule.

In Part I, however, it was postulated that secretion in this gland may be a combination of both holocrine and merocrine secretion, the extent of each depending on the type of stimulus provided. To test this hypothesis, it was decided to repeat a portion of Hirsch and Jacobs' enzyme analyses after feeding to determine (1) if rhythmic activity could be observed in O. virilis, and (2) how such cycling might be affected by a mitotic blocking agent.

Only one enzyme, a trypsin-like, casein-protease, would be studied but assays would be made every 30 minutes for $6\frac{1}{2}$ hours as in the original work.

Hirsch and Jacobs found that the first mitotic rate maximum occurred $1\frac{1}{2}$ hours after feeding and this was succeeded by maxima of E-, F-, and B-cells, the B-cell peak occurring at 4.5 hours, and by a tissue enzyme maximum at 5-5.5 hours. During the $6\frac{1}{2}$ hour period, evidence of three overlapping sets of peaks were observed: (1) the first set does not include a mitotic peak since the cells divided during starvation but the cell-type peaks are all present followed by a tissue enzyme maximum at 2.5 hours after feeding; (2) the second set, extending from 1.5 to 5.5

hours, was the only complete sequence; and (3) the last set of maxima began with a mitotic peak at about 3 hours but does not include the enzyme peak which occurred, supposedly, beyond the $6\frac{1}{2}$ hour period of observation. Thus, tissue enzyme peaks for two sequences may be found in the $6\frac{1}{2}$ hour period, the first at 2.5 hours and the second at about 5.5 hours. In the authors' view, each set of maxima represented the synchronized (due to starvation-feeding) transformation of a mass of cells from one stage to the next in their differentiation from cell division to holocrine secretion; i.e., each set is a separate wave of such synchronized cells.

If, in the present study, rhythmic protease activity is observed in O. virilis and if Hirsch and Jacobs' hypothesis is correct, prior colchicine treatment should in some way retard the cycling. However, what form should this retardation take? Theoretically, it should only affect rhythmic cycling related to, or occurring after, the first synchronous mitotic division in the presence of colchicine. If this division occurs 1.5 hours after feeding, the enzyme peak to be affected by the drug is the one normally occurring at 5-5.5 hours. Therefore, the results expected in this study, if exclusively holocrine secretion occurs in the tubule, is that, in the first half of the $6\frac{1}{2}$ hour period (locus of first enzyme peak) there will be no difference in

protease activity between controls and colchicine-treated animals but, in the second half of the period (locus of second enzyme peak), the activity rhythm will be retarded, either in time, amplitude or both.

Before beginning this investigation, pilot studies had to be run to determine effective colchicine dosage and method of exposure to block cell division in the hepatopancreas. In addition, assays of protein concentrations were carried out on the same tissues to determine whether post-feeding rhythms could also be detected in either the protein concentration or in the calculated specific activity of the protease.

Methods of Procedure

Colchicine pilot studies

Several dozen small ($1\frac{1}{2}$ to 2 inches) crayfish of mixed species seined from streams around Lake Okoboji, Iowa, were placed on a feeding schedule in the laboratory and adapted to a tap water media. Colchicine treatment was carried out by adding known amounts of colchicine to the media using final concentrations of 0, 10^{-9} , 10^{-8} , 10^{-7} , 10^{-6} , and 10^{-5} M. Solutions were mixed from a 10^{-3} M stock solution of colchicine, which was kept in a dark bottle and refrigerated. Three separate experiments were carried out to determine correct exposure time and dosage while, at the same

time, simulating the starvation-feeding stimulus to be used in the enzyme studies. Group A was fed briefly, placed in colchicine 24 hours, and examined for mitotic figures in the hepatopancreas tubules. Group B was exposed to colchicine 24 hours after feeding, starved 9 days, fed briefly and examined after six hours. Group C was fed, starved 9 days, treated with colchicine for 24 hours, fed, and examined after six hours. Since Groups A and B produced a high mortality and mixed results, these methods of exposure were not used. A more detailed description of the method used with Group C will now be presented.

Specimens were maintained in 500 ml of media in plastic mouse cages, one cage for each colchicine concentration. Four control specimens were maintained in the colchicine-free water and three crayfish were exposed to each of the five drug concentrations. Media was changed every other day during the starvation period. Colchicine exposure began at 4:45 on the day prior to examination of the tubules for mitoses and the media was protected from strong light by covering the containers with paper and turning off the room lights. After 24 hours, each specimen was hand-fed a small piece of raw hamburger to insure eating. After six hours, the glands were removed as described in Part I and transferred to van Harreveld's solution. The connective tissue covering was removed and each lobe of the gland was divided

into 6-10 pieces and transferred to fresh solution in vials. The van Harreveld's solution was then slowly diluted to 25% normal by adding distilled water in several steps over a 30-minute period in order to initiate chromosome separation and facilitate identification of metaphase figures. All solution changes from this point were carried on in the original vial using a finely drawn suction pipette with a curved tip to avoid tubule loss or injury. Tissues were fixed 15 minutes in 3:1 acetic-alcohol and washed twice in distilled water. Most of the tissue samples were then stored for future use, leaving 2-3 pieces of hepatopancreas per crayfish for the remainder of the study. Feulgen-squashes were then prepared as follows: Vials containing the fixed tissues in distilled water were transferred to a constant temperature water bath at 60°C and time allowed for the tissues to reach this temperature. The distilled water was then replaced with pre-warmed 1 N HCl and hydrolysis carried out for 12 minutes. Operations were staggered by one minute to facilitate continuous operation. After hydrolysis, tissues were washed in three changes of distilled water and stained for two hours in the colorless, Feulgen reagent mixed according to Humason (24). Here, they gradually acquired the typical violet color which was most intense at the distal tips. Staining was followed by three, two-minute periods in bleach solution (24) and tubules were

then transferred to a watch glass of distilled water for a 10-minute washing. During this step, individual tubules were freed from the gland pieces utilizing a dissecting microscope and fine needles. Five tubules from a single crayfish were transferred to a microscope slide, excess water was drawn off, and a drop of 45% acetic acid was added to soften the tubules. After two or three minutes, the tubules were examined for adequate separation on the slide, a cover slip was added, excess acetic acid was blotted, and the squash was made by inverting the slide on paper towel-ling and tapping it with a wooden probe. Tubules were located under low power and metaphase figures were identified and counted under oil immersion. Five tubules were examined from each specimen yielding 15 counts per colchicine concentration.

Enzyme assays

Approximately 100 crayfish were used in pilot studies and assays to measure the activities of pH 7.6 casein-protease and the concentrations of biuret protein in the hepatopancreas of starved and fed animals every 30 minutes after feeding. Data was collected from 84 animals including 68 specimens of Orconectes virilis and 16 of Orconectes immunis. The former were approximately 4" long and obtained from the E. G. Steinhilber Co., Oshkosh, Wisc. during

late summer after the major molting season. O. immunis was smaller in size and obtained during July from the Carolina Biological Supply House, Elon, N. C. O. virilis appeared to be in a weakened condition, possibly due to the proximity of the molting season. Two previous shipments were lost, either in toto following shipment or gradually, after a few week's time in the laboratory. The third shipment was allowed to equilibrate in the laboratory for about two weeks and time allowed for the dying specimens to be weeded out. During this time the crayfish were maintained in shallow, artificial spring water, 12 to a large plastic tub. It was found that the mortality rate decreased if a layer of coarse pebbles was placed on the bottom of the tub. The room was air-conditioned with a temperature range of about 18-21°C. On the day after their arrival, the animals were placed on a feeding regime which provided a small amount of hamburger mixed with oatmeal every three days. Those animals either still in molt or fatally weakened by it refused food and moved about sluggishly; and, each day more of their number died or were killed and partially consumed by the healthier specimens. Because of the loss of so many specimens of O. virilis, 16 out of 84 experimental points had to be filled with the second species, O. immunis. After two weeks, practically all of the remaining crayfish appeared to be in good health. Later, during the experiments, even some of

these refused the stimulus meal or required several minutes of coaxing and a few were found to have a second shell growing under the old and were thus in pre-molt.

In preparation for the enzyme studies, a schedule was followed which allowed 9-12 days of starvation. Colchicine treatment was carried out during the final day by isolating the animals in individual containers of pond water, with or without colchicine. This was always done at noon on the day preceding the assays to avoid any differences in diurnal cycling of the mitotic rate as suggested by Eigsti and Dustin on p. 375 (8). The $6\frac{1}{2}$ hour time period under investigation consisted of 14 half-hour intervals from point 0 (starved animals) to point 14 ($6\frac{1}{2}$ hours post-feeding). At each interval a total of six animals, one control-treated pair for each of three trials, were studied. Members of a pair were matched for species and size but not sex. Forty-two such pairs were numbered 1A through 14C to designate time interval and trial. The numbers were then randomized before scheduling the enzyme assays to minimize similarities between particular time intervals examined on any two days of the study.

On the day of the enzyme assay, controls were fed at 9:00 a.m., treated specimens at 9:15, and tissues collected at appropriate times after feeding. Thus, treated specimens were exposed to colchicine for 21 hours prior to feeding and

for 21-27 hours before being sacrificed. The 15-minute feeding differential allowed nearly simultaneous collection of glands from the members of a pair but yet allowed sufficient time for tissue preparation of each.

Since "time after feeding" was a crucial variable, it will be defined as the interval between the crayfish's acceptance of food and the cessation of enzymatic activities in the hepatopancreas by cold temperature, homogenization and dilution.

A few minutes prior to the scheduled time, tissue collection was initiated by cutting away the rostral portion of the crayfish's carapace and all its appendages to facilitate exsanguination as well as handling. The entire dorsal exoskeleton of the cephalothorax was then removed, and the stomach, midgut and both sides of the hepatopancreas were freed and transferred to crayfish perfusion fluid as described in Part I. The entire right hepatopancreas was then separated from the midgut, rinsed in fresh perfusion fluid, quickly blotted on filter paper, and weighed on a torsion balance to the nearest 2 mg. A 40-ml glass homogenizer containing 1 ml 10^{-3} N HCl had been placed in crushed ice and water beforehand to achieve rapid cooling. HCl was used as the homogenizing medium to retard autoactivation and digestion of the protease. After being weighed, the tissue was transferred to the bottom of the homogenizer and crudely

broken up with the pestle to facilitate cooling. The amount of 10^{-3}N HCl necessary to yield a 5% w/v homogenate was quickly calculated, 1 ml was subtracted, and the resulting volume added to the homogenizer. Homogenization was then carried out in the ice bath with 15 strokes of the pestle. Approximately 30 seconds elapsed between transfer of the gland to the homogenizer tube and commencement of homogenization; thus, "time after feeding" is probably accurate to within one minute on graphs in the Results section.

The homogenate was then transferred to a 15-ml centrifuge tube, centrifuged in a table model centrifuge at half speed (about 2500 rpm) for 10 minutes and the centrifugate transferred to a pre-chilled test tube and stored at $5-9^{\circ}\text{C}$ for 1 to 6 hours until the remainder of the samples had been collected.

Assay of the casein-protease was carried out according to a modification of Kunitz' method for assaying trypsin activity (31). Pilot studies showed a linear increase in optical density with increased volume of homogenate up to 0.3 ml. The procedure was carried out in 15-ml centrifuge tubes at 35°C in the following steps:

1. Add 0.9 ml 0.1 M Sorensen's phosphate buffer, pH 7.6.
2. Add 0.1 ml, well-shaken, 5% homogenate centrifugate.

3. Timing this step to the second, add 1 ml substrate (1% casein in buffer).
4. After exactly 20 minutes, precipitate remaining protein with 3 ml 10% TCA and mix well with glass stirrer.
5. Allow to stand one hour.
6. Filter through Whatman #1 filter paper into clean test tube.
7. Read O.D. at 280 millimicra against blank.

For each sample, three assay tubes were prepared including two experimental and one blank. Thus, each sample was used to make its own blank by adding TCA prior to the substrate. Optical densities were read in a Beckman DB spectrophotometer using the narrow slit and a hydrogen UV light source. Samples were placed in quartz cuvettes, 100% transmission was adjusted for the blank, the two experimental tubes were read and their average was taken as the O.D. for the specimen.

Modifications of Kunitz' procedure were as follows:

(1) TCA precipitate was removed by filtration rather than centrifugation because of the presence of floating debris from the precipitate which could not be spun down even with higher speeds. (2) A standard curve was not constructed because relative values were adequate to observe rhythmic fluctuations in activity and, since neither the nature of

the protease nor that of its casein hydrolysate is defined, even a standard curve would yield relative values. Enzyme activity was expressed directly in terms of optical density, i.e. the difference in O.D. at 280 millimicra between experimental and blank samples per 0.1 ml homogenate per 20 minutes ($\Delta O.D.^{280}/0.1 \text{ ml}/20 \text{ min}$). (3) Blanks were prepared for each sample. They were especially critical because of the variation in pigment and coloration among the glands and the high U.V. absorption of TCA.

The remainder of the homogenate was shaken to resuspend its contents and about 5 ml were stored in a vial at -30°C for the protein assays.

Protein assays

The high concentration and variety of lipids in the hepatopancreas yielded a turbid and colored homogenate centrifugate with suspended lipid droplets and pigments which would interfere with colorimetric protein assays. TCA precipitation of the homogenate yielded a cream to greenish precipitate and a supernatant with yellow-green oil droplets and other debris, either floating on the surface or sticking to the sides of the tube. These conditions persist in the solution through the various steps preparing it for reading in the colorimeter and thus introduce substantial errors in the absorptions. The method finally employed was a modifi-

cation of the technique of Hiller, McIntosh and Van Slyke for assaying urine protein using a NaOH-CuSO₄ biuret reaction and to which lipid extraction steps were added for the present study. The original method is described in Hawk, Oser and Summerson (18).

Frozen samples were thawed and vigorously shaken to resuspend lipid droplets and other debris. The homogenate was drawn up and down in the pipette several times before measuring a sample. Details of the modified method are as follows:

1. 1 ml of homogenate centrifugate is transferred to a 15 ml centrifuge tube.
2. 1 ml of 10% TCA is added to homogenate with vigorous stirring; allowed to stand 15 minutes.
3. Mixture is centrifuged 10 minutes at #60 (about 3000 rpm) in table model centrifuge.
4. TCA is decanted and discarded; tube is drained by inverting on paper towel for 5 min.
5. Pellet is extracted with 3 ml 2:1 ether:ethanol (all oily debris adhering to sides of tube is removed with this step); drain on paper towel.
6. Extraction is repeated twice for 20 min each time.
7. Pellet is broken up in 1.00 ml 3% NaOH using a thin glass rod and yields a turbid, beige-colored fluid.

8. Gradually add 2.00 ml of 3% NaOH in steps with mixing and standing between additions to facilitate dissolving of the protein.
9. Add 0.10 ml of 20% CuSO_4 and mix vigorously; allow to stand exactly 15 min. A turbid, blue to violet solution with a blue, crystalline ppt. will appear.
10. Centrifuge exactly 15 min at #60. A clear solution with some debris floating in a very compact layer on the surface should result.
11. Clean surface with a finely-drawn glass suction pipette with curved tip. Even extensive cleaning resulted in less than 0.5 ml fluid loss out of 3 ml.
12. Read absorption of the supernatant against a blank of 3 ml of 3% NaOH and 0.1 ml CuSO_4 at 540 millimicra. Samples were read quickly in separate cuvettes since a crusty film is left by the NaOH which isn't removed by simple rinsing.

For the standard curve a stock, 0.5% solution of purified casein in the above buffer was prepared and used to make up a range of % casein solutions including 0.10, 0.25, 0.30, 0.35, and 0.40%. The above assay procedure was carried out on these protein solutions and yielded a standard curve which was linear over the range 0 to 0.5% protein. This corresponded to an O.D. range of 0 to 0.288. The

O.D.'s of the homogenates were in the range 0.10 to 0.20 which corresponded to homogenate protein concentrations of 0.15 to 0.5%. Since the amount of protein lost during centrifugation of the homogenates was not measured a comparison with normal protein values in the hepatopancreas can only point out similarities in range. The above measurements indicate gland protein concentrations from 3% to 10% (plus the amount lost in centrifugation). Literature values for one crab, Cancer pagurus, range from 3.00% to 6.22% (56).

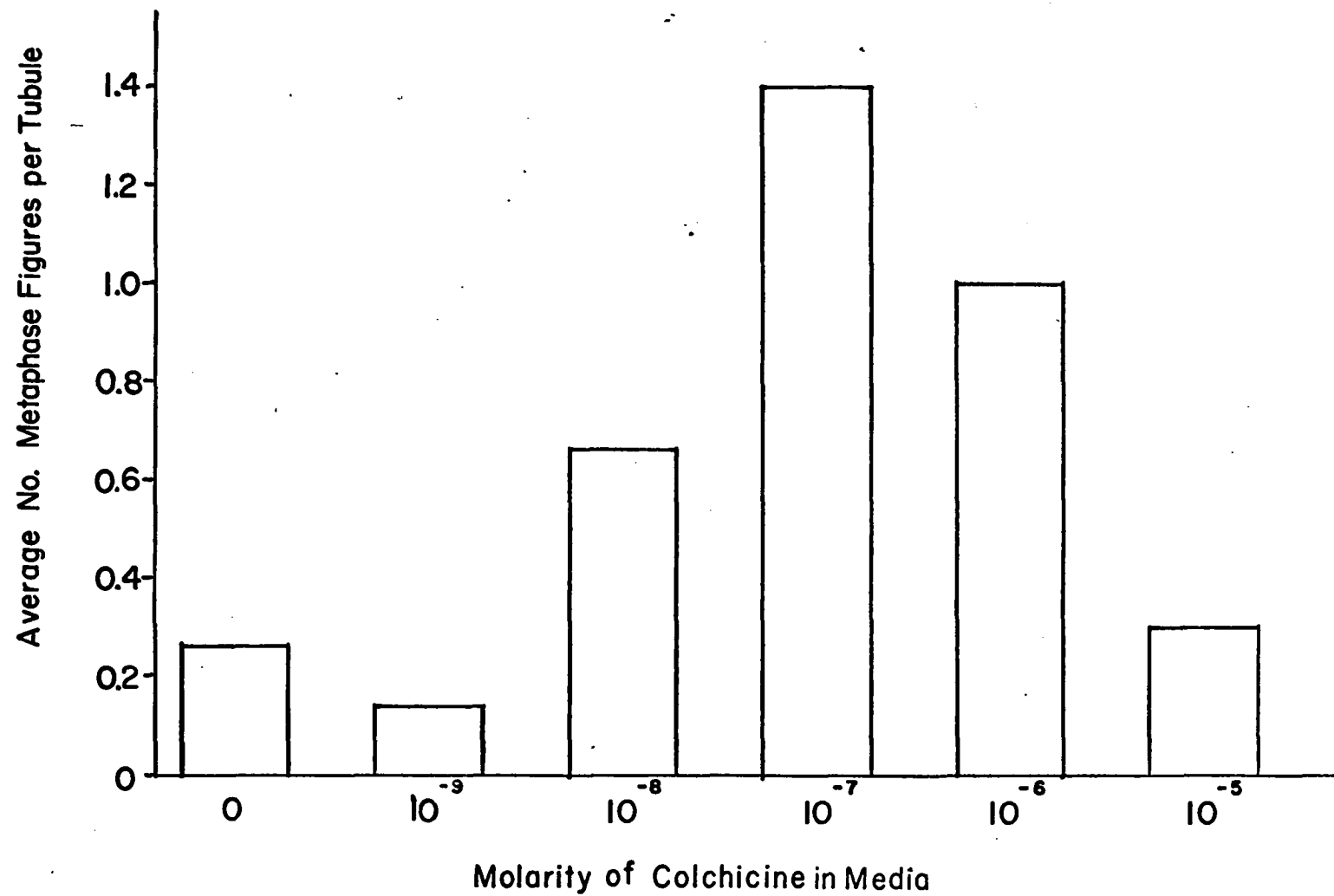
Results

Colchicine studies

It was mentioned above, that Groups A and B showed a high mortality rate and did not yield reproducible results while, in Group C, no specimens died although one animal appeared to be diseased and three glands did not appear healthy.

The results obtained with Group C are shown in Fig. 36. In the squash preparations, the five tubules per slide were well spaced and easy to locate. Various regions of the tubule were easily differentiated because cells remained orientated though spread and because the distal portion was distinguishable by a freedom from fat droplets and the presence of small, compact cells. These aids made counterstain-

Fig. 36. Histogram showing effects of colchicine dosage on mitotic arrest
Maximum number of metaphase figures per tubule occurred with $10^{-7}M$
colchicine. Drug was administered by exposing intact animal to
colchicine in water for 24 hours.



ing unnecessary. All nuclei stood out as bright, violet-colored structures against the light, yellow green background of the tubule cells. Mature tubule cells possessed very large, ellipsoidal nuclei with wrinkled surfaces; connective tissue and muscle cells had very small, dense and flat nuclei; and the cells in the blind tip were of intermediate size and density and were spherical in shape. Mitotic figures were easily identified under oil immersion and appeared to possess the very high chromosome number (circa 200 for $2n$) reported for several crustaceans by Makino (36) although no attempt was made to count the chromosomes. Because of the high number and minute size of the latter, it was difficult to identify a few figures as being either late prophase or metaphase and, in these instances, the figure was not included.

The number of definite metaphase figures ranged from 0 to 5 per tubule yielding average counts from 0.13 to 1.4. Fig. 36 demonstrates that a normal curve results when average counts are graphed against colchicine concentrations between 0 and $10^{-5}M$. The highest number of figures was obtained with $10^{-7}M$, while 10^{-9} and $10^{-5}M$ colchicine yielded counts about the same as control animals which were not exposed to the drug.

In addition to these quantitative results, qualitative changes were observed with the higher colchicine concentra-

tions. With $10^{-7}M$ the chromosomes in a small number of cells appeared as a clumped mass. The number of such figures rose sharply with $10^{-6}M$ and remained high with $10^{-5}M$. In addition, the latter two concentrations seemed to alter the general appearance of cells in the distal end of the tubule; the nuclei appeared more angular, as though packed together more tightly, the tip itself appeared larger and more bulbous than normal and, although cell counts were not made, there appeared to be more cells in this region compared to the situation with lower colchicine concentrations.

For these reasons, it was decided to use $10^{-7}M$ colchicine in the enzyme studies.

Casein-protease assays

The activities of pH 7.6 casein-protease for all 84 specimens are given in Table 1 and Fig. 37. When the data are examined without regard to species or molting state, the differences in O.D. per 0.1 ml per 20 minutes ranged from 0.048 to 0.204 for control specimens and from 0.048 to 0.290 for treated specimens. The results for both groups display a great deal of scatter and the probable reasons for this will be analyzed in the Discussion. In general, both groups show signs of a bimodal distribution of activities which is more evident in the controls; that is, the values appear to be grouped into two maxima which are separated by

Table 1. Activities of casein-protease at intervals after feeding

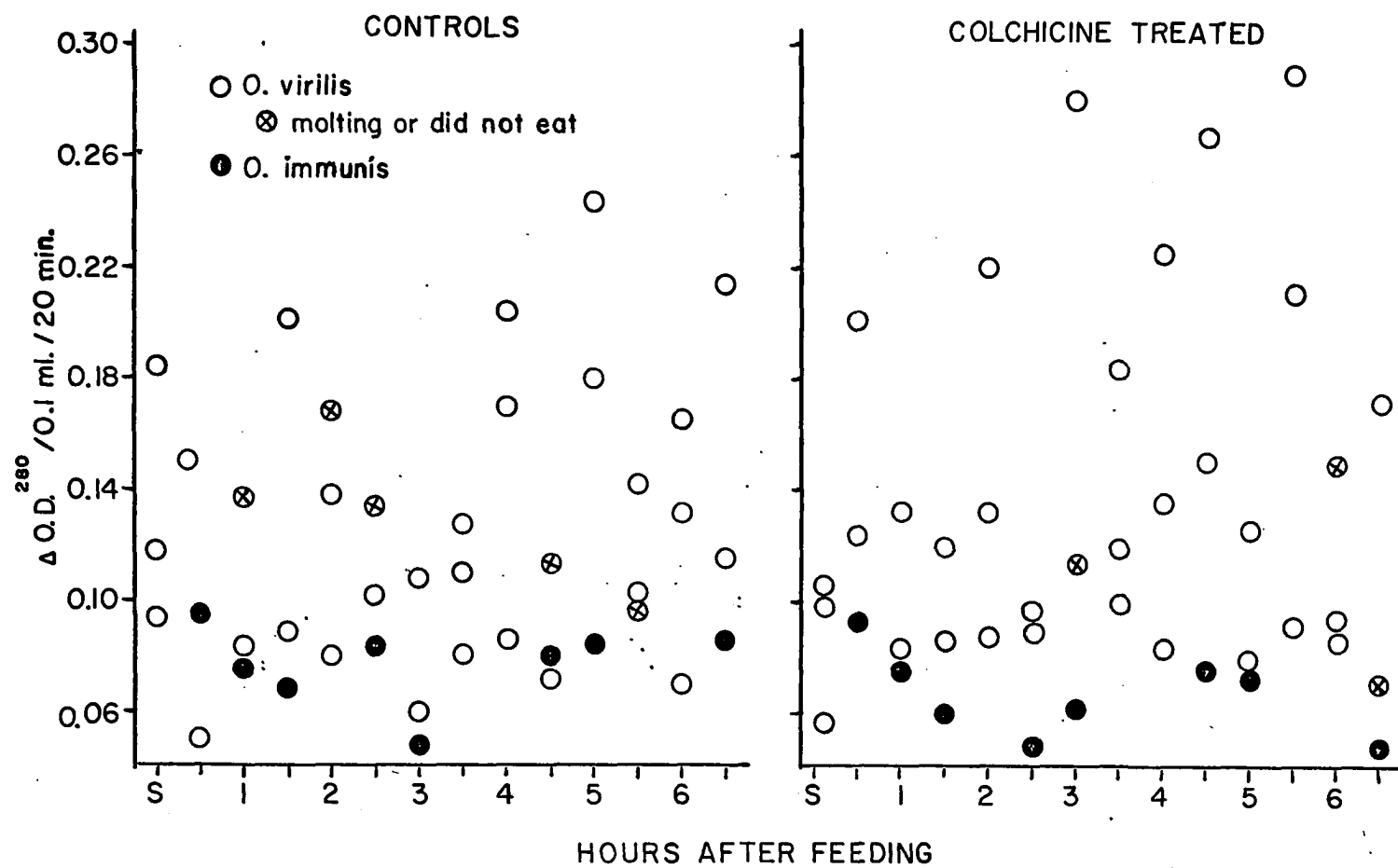
Hours	$\Delta O.D.^{280}/0.1 \text{ ml}/20 \text{ min}$							
	Controls				Colchicine-treated			
	A	B	C	Average	A	B	C	Average
Starved	.118	.094	.184	.132	.100	.056	.103	.086
0.5	.050	.150	.095 ^a	.098	.201	.124	.093 ^a	.139
1.0	.082	.137 ^b	.079 ^a	.099	.132	.082	.078 ^a	.097
1.5	.201	.089	.068 ^a	.119	.086	.120	.060 ^a	.089
2.0	.080	.168 ^b	.138	.129	.088	.221	.132	.147
2.5	.102	.134 ^c	.084 ^a	.107	.096	.090	.048 ^a	.078
3.0	.108	.060	.048 ^a	.072	.114 ^b	.281	.062 ^a	.152
3.5	.081	.128	.110	.106	.100	.184	.120	.135
4.0	.086	.204	.170	.153	.084	.226	.136	.149
4.5	.072	.114 ^c	.080 ^a	.089	.151	.268	.076 ^a	.165
5.0	.180	.243	.085 ^a	.169	.126	.080	.072 ^a	.093
5.5	.142	.102	.098	.114	.092	.211	.290	.198
6.0	.070	.132	.165	.122	.088	.150 ^b	.093	.110
6.5	.116	.214	.086 ^a	.139	.072 ^b	.172	.048 ^a	.097

^aQ. immunis.

^bDid not eat stimulus meal.

^cMolting.

Fig. 37. Graph of protease activity in hepatopancreas after feeding
Scatter plot demonstrated wide variation among the 84 animals
assayed. O. immunis is consistently lower than O. virilis.
Bimodal distribution is more evident in controls than in
colchicine-treated group.



a minimum near the middle of the $6\frac{1}{2}$ hour period. For the controls, this minimum occurs at about 3 hours after feeding and, for the treated specimens, at about $2\frac{1}{2}$ hours. The activities from O. immunis are consistently lower than those of O. virilis but the tendency toward two maxima is seen here also. If the enzyme activities in the two halves of each curve are averaged independently, the following values are obtained:

<u>Time after feeding:</u>	<u>0-3 hrs.</u>	<u>$3\frac{1}{2}$-$6\frac{1}{2}$ hrs.</u>
Controls:	0.108	0.128
Treated:	0.113	0.135

Thus, the average protease activity of controls and treated are essentially the same in each half of the period and activity is about 20% higher for the second half of the $6\frac{1}{2}$ hour period. Before more detailed analyses could be undertaken, it was necessary to formulate criteria for the elimination of doubtful values in order to reduce the scatter. Data selection precluded the use of statistical analyses later to determine significant differences due both to the reduction in sample numbers and the possible introduction of bias. However, the criteria could be justified by factors other than the data, itself, and this was the only means for detecting subtle changes in enzyme activity with time. The following criteria for acceptable specimens were adopted:

1. Only values for O. virilis would be included. In

addition to being another species, O. immunis had consistently lower protease activities indicating it could not be used to represent the same populations as O. virilis.

2. Specimens obviously in molt were eliminated. Because of the central role of the hepatopancreas in molting and the degenerative effects of molting on the gland's metabolism, animals in this state could not be considered normal. No attempt was made to identify more subtle signs of molt stage.

3. Specimens which refused food or required several minutes of coaxing before eating were excluded. Feeding was the stimulus for the entire experiment and these conditions either made the results worthless or introduced errors in "time after feeding." Also, crayfish starved for 9-12 days usually do not refuse food unless they are diseased or in molt.

4. A time interval was left blank unless values from at least two specimens were available. The normal range of values was considered too wide to allow comparison of a single activity with averages of two or three.

The following is a breakdown of the number of animals eliminated by these criteria:

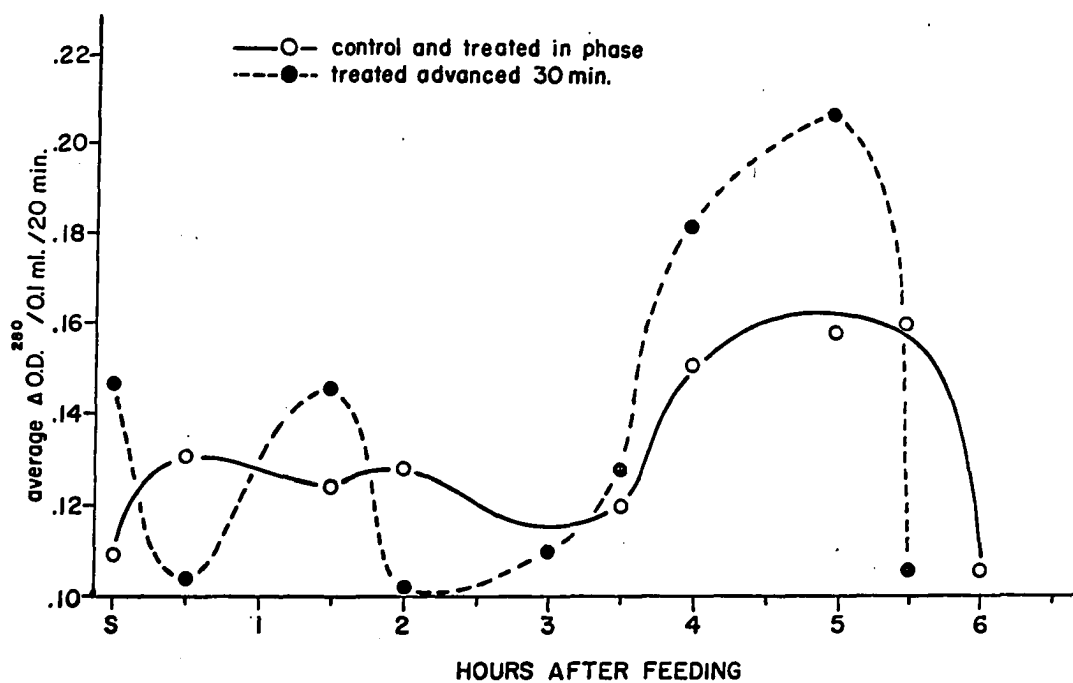
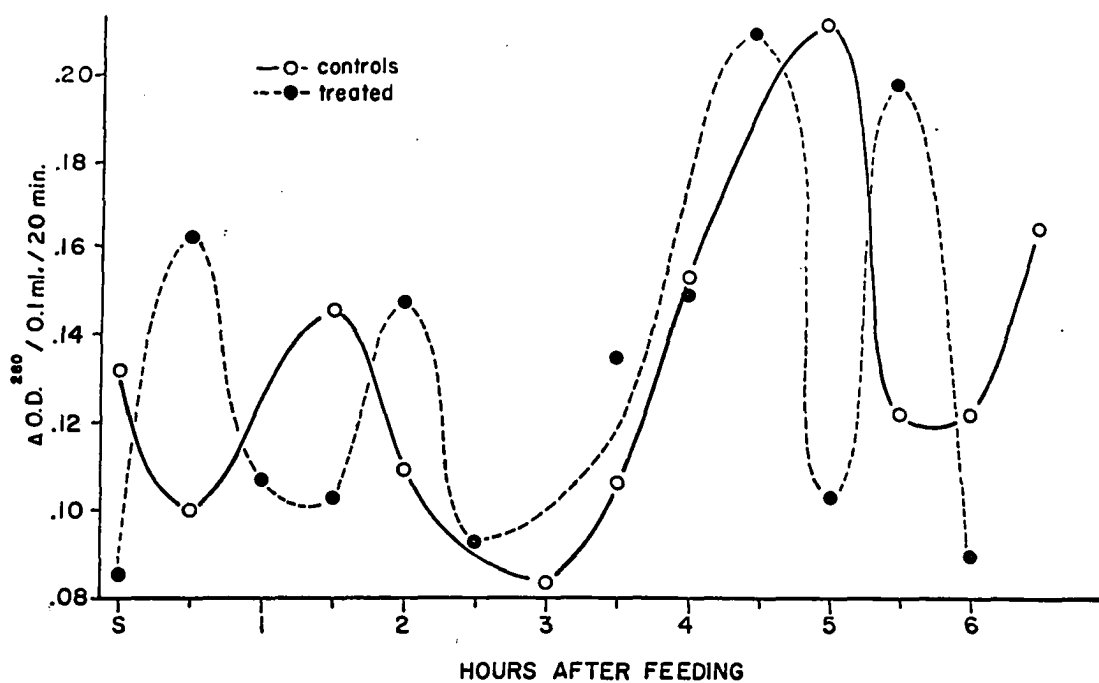
	<u>Controls</u>	<u>Treated</u>
Original number	42	42
Number eliminated by		
criteria #1	8	8
criteria #2	2	0
criteria #3	2	3
criteria #4	<u>3</u>	<u>2</u>
Total number eliminated	15	13
Final number included	27	29

In all further analyses, data from animals in the last group will be referred to as the "selected values" as opposed to the 84, original values.

When the averages of the selected values of casein-protease activity are plotted against time after feeding the general trends observed in the scatter plots of the original values become much more apparent. In Fig. 38 the control enzyme activities are seen to decrease during the first half hour and then increase, yielding a maximum at $1\frac{1}{2}$ hours. Activity then decreases to its lowest point at 3 hours. A second maximum, nearly twice the value of the first, appears at 5 hours, after which time activity decreases and then begins to rise again at the end of the period studied. Glands from colchicine-treated animals start at low activity which immediately increases to a first maximum at $\frac{1}{2}$ hour, then decreases to a minimum at $1-1\frac{1}{2}$ hours. A second maximum appears at 2 hours and then a drop similar to that of the controls with a minimum at $2\frac{1}{2}$ hours. The activity then

Fig. 38. (top) Rhythmic protease activity after feeding
Each dot or circle represents an average of two
or three animals. Activities of controls and
colchicine-treated animals are similar in values
but appear out of phase.

Fig. 39. (bottom) Phase-check of protease activity curves
Each dot or circle represents the average of a
control average and a treated average in the
above graph. See text for explanation.



rises to a high maximum at $4\frac{1}{2}$ hours, again similar to the controls, followed by a minimum at 5 hours, a maximum at $5\frac{1}{2}$ hours and a drop to a very low value at 6 hours.

Because the two curves displayed similarities in values but differences in timing, efforts were made to examine the curves for phase differences in their rhythmic fluctuations. By averaging the control and treated values at each time interval, it could be ascertained whether maximum and minimum values were increased, decreased or unaltered by the addition and whether or not the general smoothness of the curves was improved. This information could give indications as to the general phase agreement between control and treated curves in a manner analogous to constructive and destructive interference of light waves.

Fig. 39 shows the results of such a phase check on the above enzyme activity data. When control and treated values from the same time interval are simply averaged together and the results plotted against time after feeding, most of the rhythmic variation seen in the separate curves is lost.

There is an initial increase in activity at $\frac{1}{2}$ hour, a nearly stable activity for the next 3 hours and a broad maximum during the second half of the curve with a final drop at 6 hours. However, if the treated values are first advanced 30 minutes and then averaged with the controls, a smooth rhythmicity results which enhances that in the separate

curves; there is an initial decrease in activity to a minimum at $\frac{1}{2}$ hour, a maximum at $1\frac{1}{2}$ hours, a minimum at 2-3 hours, a high broad maximum reaching its peak at 5 hours and a final drop at $5\frac{1}{2}$ hours.

Protein assays

Results of the biuret-protein assays in hepatopancreas homogenates from 83 specimens are given in Table 2. Accidentally, one sample was used up in standardizing the protein assay technique. The protein concentrations varied from 1.45 to 3.45 mg/ml for the controls and 1.17 to 3.52 mg/ml for treated specimens. Fig. 40 shows a plot of selected values against time after feeding. In general, the control curve reaches higher values and undergoes greater changes in amplitude than that of the treated. In spite of these differences, there is a striking correlation between the maxima and minima of the two curves; that is, the control and treated values differ in quantity but their fluctuations are synchronized. Another observation is that values in the second half of the curve do not increase over those in the first as seen in the enzyme curves.

These relationships are made more apparent in Fig. 41 which shows a phase check of this data. The "in-phase" averages yield a smooth curve with time beginning with an initial decrease in protein during the first $\frac{1}{2}$ hour followed

Table 2. Concentrations of biuret protein at intervals after feeding

Hours	mg protein/ml							
	Controls				Colchicine-treated			
	A	B	C	Average	A	B	C	Average
Starved	1.68	2.70	2.50	2.29	2.42	3.52	- ^a	2.97
0.5	2.18	2.52	2.47 ^b	2.39	1.17	2.52	2.25 ^b	1.98
1.0	3.18	2.48 ^c	1.98 ^b	2.55	2.75	2.58	1.30 ^b	2.21
1.5	3.40	3.35	2.20 ^b	2.98	2.98	2.43	2.12 ^b	2.51
2.0	1.58	2.17 ^c	3.07	2.27	2.38	2.68	2.35	2.47
2.5	2.50	2.86 ^d	1.77 ^b	2.38	2.30	3.42	1.77 ^b	2.50
3.0	3.05	1.88	1.80 ^b	2.24	2.70 ^c	2.75	2.23 ^b	2.56
3.5	2.84	3.49	2.33	2.89	3.17	2.33	2.50	2.67
4.0	2.34	2.72	1.45	2.17	1.87	2.62	1.82	2.10
4.5	2.03	1.95 ^d	1.88 ^b	1.95	2.34	2.74	1.98 ^b	2.35
5.0	3.27	3.28	2.07 ^b	2.87	2.72	2.62	1.72 ^b	2.35
5.5	1.90	1.97	1.84	1.90	1.97	2.42	2.78	2.39
6.0	2.34	2.36	2.32	2.34	2.00	3.10 ^c	2.02	2.37
6.5	3.16	2.90	1.58 ^b	2.55	2.58 ^c	2.08	1.90 ^b	2.19

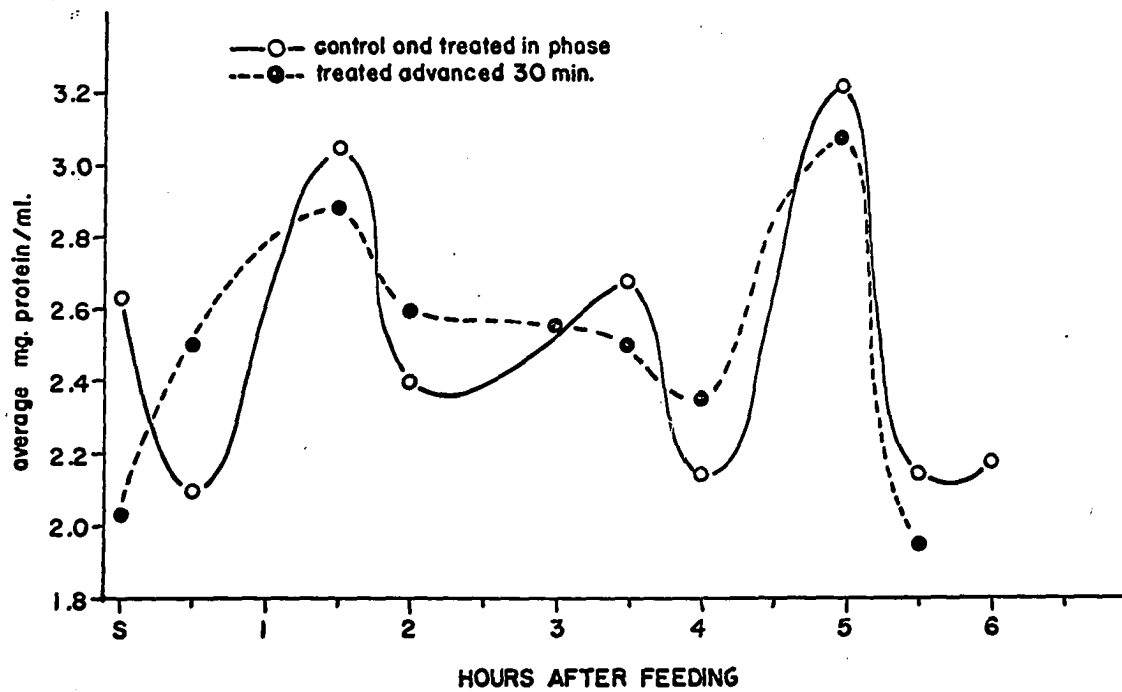
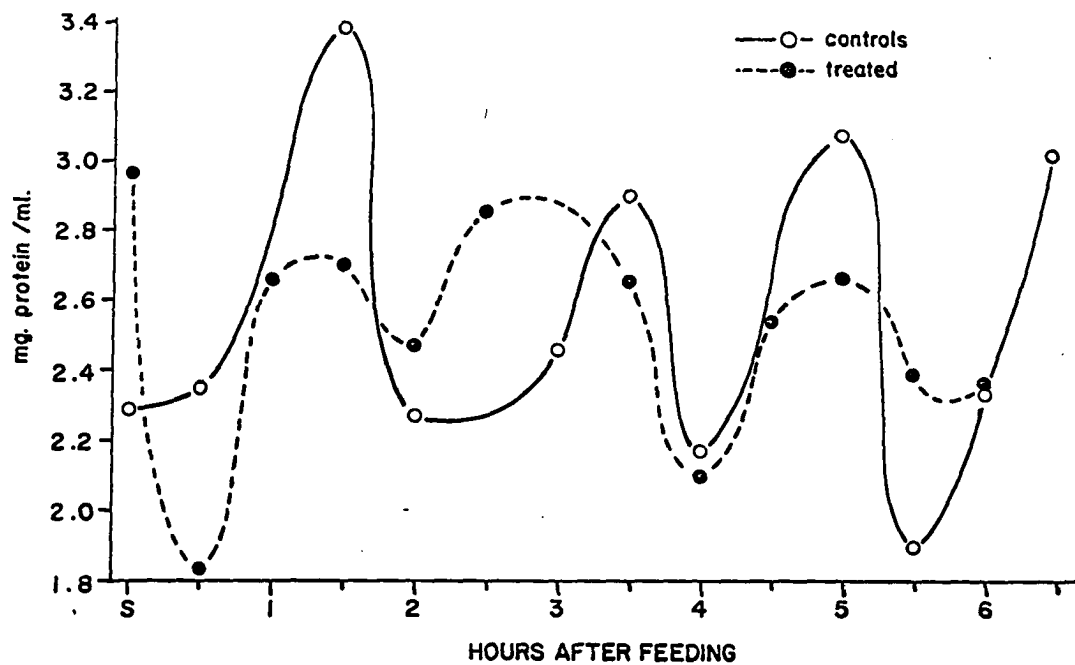
^aSample used up before assay.

^bO. immunis.

^cDid not eat stimulus meal.

^dMolting.

- Fig. 40. (top) Rhythmic protein concentrations after feeding
Same criteria as Fig. 38. Control and treated values appear to be of unequal magnitudes but their fluctuations are in phase.
- Fig. 41. (bottom) Phase-check of protein concentration curves
Same criteria as Fig. 39.



by maxima at $1\frac{1}{2}$, $3\frac{1}{2}$ and 5 hours after feeding. When the treated values are advanced 30 minutes before averaging, there is a decrease in both the absolute values and the amplitude of the fluctuations. The protein concentration increases to a maximum at $1\frac{1}{2}$ hours, remains more or less steady between 2 and 4 hours with a slight decrease at 4 hours. This is followed by a second maximum at 5 hours and a decrease at $5\frac{1}{2}$ hours to about the same value as the controls.

Specific enzyme activity

Specific activity was calculated by dividing each enzyme activity by its respective protein concentration and was expressed as the difference in optical density per mg protein per 20 minutes ($\Delta O.D.^{280}/mg/20 \text{ min}$). Table 3 gives the specific activity for 83 specimens. The values ranged from 0.229 to 1.172 for controls and from 0.159 to 1.718 for treated specimens.

Fig. 42 shows the curves for selected values. Specific activities in control specimens showed little variation during the first half of the curve beginning with an initial decrease to a minimum at $\frac{1}{2}$ -1 hour, a low maximum at 2 hours and a minimum at 3 hours. The second half of the curve shows an activity about twice that of the first half beginning with a steep rise to a maximum at 4 hours followed by

Table 3. Specific activities of casein-protease at intervals after feeding

Hours	Δ O.D. ²⁸⁰ /mg/20 min							
	Controls				Colchicine-treated			
	A	B	C	Average	A	B	C	Average
Starved	.702	.348	.736	.595	.413	.159	- a	.286
0.5	.229	.595	.385 ^b	.403	1.718	.492	.413 ^b	.874
1.0	.258	.552 ^c	.399 ^b	.403	.480	.318	.600 ^b	.466
1.5	.591	.266	.309 ^b	.389	.288	.494	.283 ^b	.355
2.0	.506	.774 ^c	.450	.577	.370	.825	.562	.586
2.5	.408	.468 ^d	.474 ^b	.450	.417	.263	.271 ^b	.317
3.0	.354	.319	.267 ^b	.313	.422 ^c	1.022	.278 ^b	.574
3.5	.285	.368	.472	.375	.315	.790	.480	.528
4.0	.368	.750	1.172	.763	.449	.862	.747	.686
4.5	.355	.585 ^d	.426 ^b	.455	.645	.978	.384 ^b	.669
5.0	.550	.741	.411 ^b	.567	.463	.305	.419 ^b	.396
5.5	.747	.518	.533	.599	.467	.872	1.043	.794
6.0	.299	.559	.711	.523	.440	.484 ^c	.460	.461
6.5	.367	.734	.544 ^b	.548	.279 ^c	.827	.253 ^b	.453

^aSample used up before protein assay.

^b0. immunis.

^cDid not eat stimulus meal.

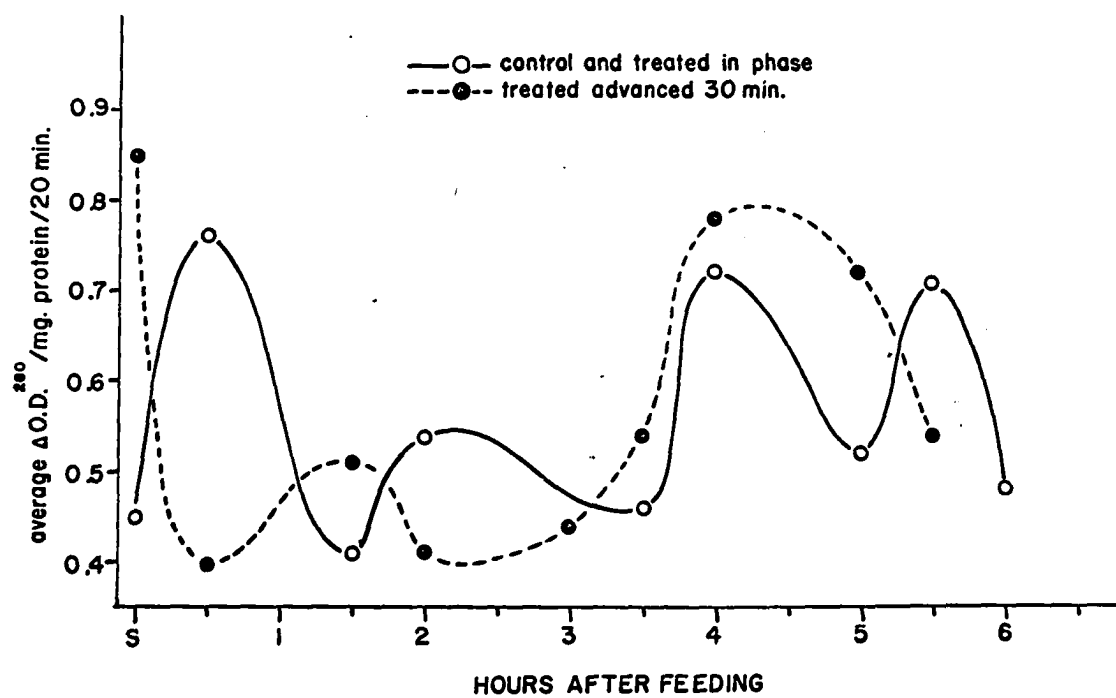
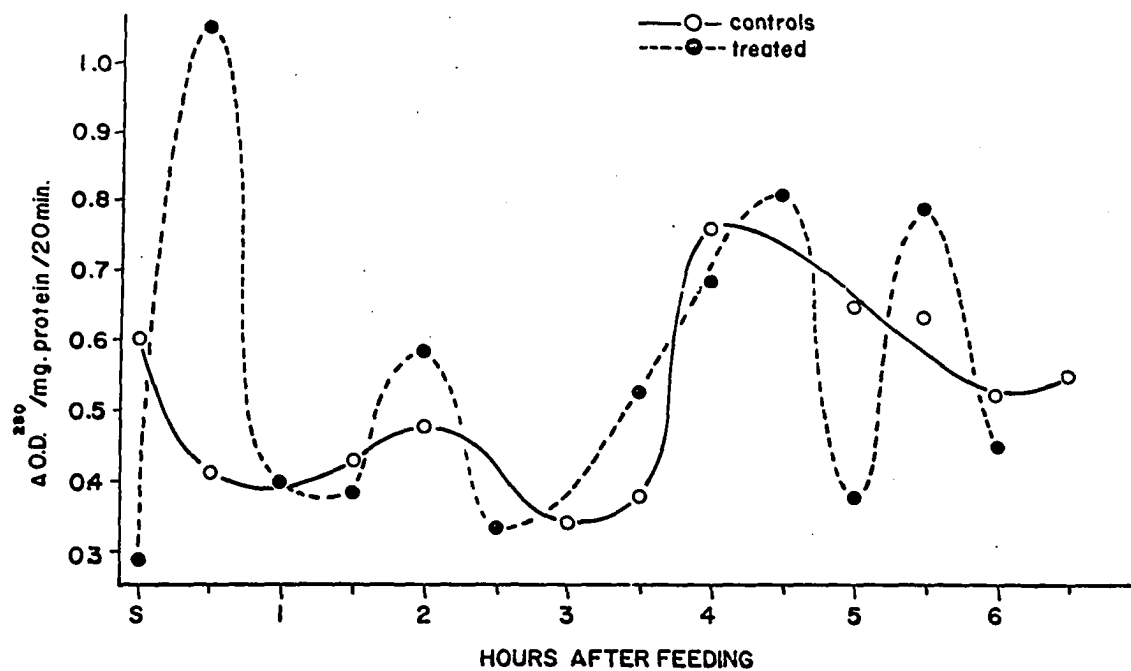
^dMolting.

Fig. 42. (top) Rhythmic protease specific activities
after feeding

Same criteria as Fig. 38.

Fig. 43. (bottom) Phase check of protease specific
activities curves

Same criteria as Fig. 39.



a slow descent from 4 to $6\frac{1}{2}$ hours. In general, the entire curve is smooth and possesses broad maxima and minima. In contrast, the treated curve has four maxima, at $\frac{1}{2}$, 2, $4\frac{1}{2}$ and $5\frac{1}{2}$ hours and undergoes rapid fluctuations beginning with a very steep rise during the first 30 minutes.

A phase check of the specific enzyme activity data, shown in Fig. 43, gives the following results: "in-phase" averaging of control and treated values yields a curve which initially rises, contains four maxima, and then drops. The points appear randomly distributed, simply alternating as maxima and minima of various heights indicating very frequent and rapid changes in activity. Advancing the treated values by 30 minutes before averaging with control values yields a smooth curve which drops sharply during the first 30 minutes, rises to a maximum at $1\frac{1}{2}$ hours, undergoes a minimum at 2-3 hours followed by a broad, second maximum at 4-5 hours which is higher than the first, and finally drops down at $5\frac{1}{2}$ hours.

Discussion

Colchicine dosage

A concentration of 10^{-7} M colchicine yielded the highest number of mitotic figures of the five doses tested. It was assumed, therefore, that this dosage effectively blocked mitotic division in the distal tip of the hepatopancreas tu-

bule when administered in the manner described. It must be pointed out, however, that this assumption was not based on reported values in the literature but had to be made on the basis of general information on colchicine treatment which makes the effectiveness of this concentration seem likely. Most of the basic information on colchicine action was obtained from Eigsti and Dustin (8). (1) A dosage between 10^{-8} and $10^{-5}M$ appears effective for most species and tissues. Therefore, the experimental range 10^{-9} to $10^{-5}M$ covered in this study should have included the most probable optimum dosage for crayfish hepatopancreas. (2) Dosages above that required to simply arrest mitosis usually produce a variety of toxic side effects within the cell. These may include, in addition to inhibition, a clumping of the chromosomes and such cells may recover but many terminate in death. The presence of clumped chromosomes seen in the present study and their increase in number above $10^{-7}M$ leads one to suspect that the higher dosages of colchicine probably exert effects beyond typical metaphase arrest which are probably toxic to the cell. (3) Observations of changes in the morphology of the distal tip reinforces this suspicion of extra-mitotic effects at the higher dosages. (4) Correct timing of colchicine exposure is undefined except for a few systems and must usually be determined empirically.

There may be a period of latency before which the drug acts, thus increasing the required exposure time; whereas, if too long a treatment is employed, recovery of the cells may already be underway before mitotic figures are counted. Many systems have an optimum six hour exposure time but this is far too short for most cold-blooded animals and even some of the homiotherms; e.g. 24 hours is the recommended exposure time for bone marrow culture. An exposure time of 24 hours was chosen in this study because (1) the crayfish is poikilothermic and (2) colchicine was administered via the pond water which meant that considerable time probably elapsed before the drug came into contact with the hepatopancreas cells, and its concentration was probably reduced by selectively permeable membranes such as the epithelium of the gill branchiae through which it probably entered the animal. Both factors would result in a far lower exposure than the apparent 24 hours in $10^{-7}M$. Therefore, the peak number of metaphase figures at $10^{-7}M$ colchicine was interpreted as being the most effective mitotic arresting of all the concentrations tested.

The number of metaphase figures per tubule was small, ranging from 0 to 5 with an average of 1.4 per tubule in $10^{-7}M$ colchicine. The results are in agreement with values of 0 to 6 per tubule reported by Hirsch and Jacobs (21). When the data of these authors is examined at each time in-

terval after feeding, the ranges were 0 to 0.5 per tubule in the summer and winter, and 0 to 2.2 in the spring.

Suggestions of extra-mitotic effects in the tubules deserves further consideration. The appearance of greater numbers of cells, more closely packed together, in the distal tip of the tubule at higher concentrations indicates either increased cell division and/or inhibition of cell migration. From the above arguments concerning dosage, increased cell division was probably not a factor in these deformities. Eigsti and Dustin (8) have discussed the ability of colchicine to produce tumors in plants. The growth of these abnormalities is not due to colchicine-induced polyploidy nor is it related to any of the mitotic effects of the drug. C-tumors, as they are called, possess the same volume as analogous tissue in the normal plant but there is a complete loss of cell polarization during tissue growth, the cells simply moving haphazardly in any direction after division. Apparently the phenomena has been studied almost exclusively in plants; however, the possibility exists that colchicine treatment may have affected cell migration in the hepatopancreas tubule. Related to this subject is the current interest in microtubules, filaments and similar linear macromolecular complexes found in many cells but most frequently in various types of movement systems possessed by cells (1). Since colchicine acts by interfering with

spindle fiber synthesis (8), an interesting approach might be to study the effects of this and other spindle poisons on various types of movement systems including those having a role in cell migration.

Enzyme assays

Sources of scatter Results of the 84 assays of protease activity were disappointing in view of the high scatter obtained. Several causes of this wide variation are now realized and most are related to the crustacean molting cycle and its widespread effects on the animal's over-all physiology.

Metabolic instability Although crayfish are always in some stage of the molt cycle, the most dramatic effects occur in pre-molt, ecdysis, and post-molt. This entire period is marked by a lack of metabolic stability. For example, the concentrations of glycogen, lipids and proteins in the hepatopancreas rapidly rise or fall and the animals eat little or no food. The specimens of O. virilis used in this experiment passed through a principal molt during early summer and were assayed in mid-August. The high mortality rate of these crayfish during and after shipment, their reluctance to eat, and the presence of a few specimens still in pre-molt, all testified to their weakened condition. Even though only healthy animals which had sur-

vived this early period in the laboratory and ate well were used in the experiments, their metabolic parameters were probably still far from stable and various activities and metabolic concentrations were probably still declining or ascending as the case may be. Thus, animals which normally would have displayed equal protease activities but were in slightly different molt stages would exhibit dissimilar activities with a resultant increased scatter of values.

Scatter due to lower values Hirsch and Jacobs

(21) showed that digestive enzyme activities in crayfish were much lower in summer and winter compared to values in the spring, both in quantity and in amplitude of fluctuation. When all of these values are graphed together, the scatter of the summer values does not appear excessive. However, when the summer values are graphed alone and the scales expanded to create amplitudes similar to spring value, the scatter appears much greater than that of the spring values. That is, the per cent variation increases as the average level of activity decreases.

In general, therefore, the scattered values reflect a highly unstable metabolic condition of the animals during the period in which they were studied. Presumably, if the same experiments were performed in the spring, the values would be higher and the scatter lower.

Effects of colchicine

In spite of the scatter, there are striking similarities in the original data between control and colchicine-treated protease activities including the averages in each half of the $6\frac{1}{2}$ hour period and the 20% rise in the second half. Thus, colchicine did not effect any apparent decrease in protease activity from the original values.

Examination of the selected value curves confirms this similarity and yields the following information: (1) Protease activities in both control and treated specimens undergo a definite cycling for $6\frac{1}{2}$ hours after feeding. (2) In general, neither the peak enzyme activities nor the intervals between peaks were appreciably altered by colchicine. (3) There appears to be a 30 minute retardation of cycling over the entire treated curve compared to the controls. That is, summing the control and treated curves causes reinforcement of maxima and minima only if the treated curve is advanced 30 minutes. The fact that this phase disagreement is present but not a decrease in activity is interesting in view of the null hypothesis of this experiment; i.e. holocrine secretion would be indicated by a reduction in protease activity during the second half of the curve.

Causes of retardation Two possibilities might explain the inducement of retardation by colchicine: (1)

secretion in the tubule is holocrine, but mitotic blocking had a different effect than simple inhibition of protease activity. Instead, the blocking merely resulted in fewer, mature secretory cells available at the time of feeding and, by some mechanism, secretion was delayed until the appropriate number of secretory cells had completed differentiation. This process required the extra 30 minutes.

(2) The second possibility is that secretion is not exclusively holocrine and retardation was due to the action of colchicine on some extra-mitotic factor(s) related to the secretion of active protease. These factors might include cell differentiation, protease synthesis, and protease extrusion among others.

The first alternative is difficult to accept because the curve indicates a true inhibition of secretion for 30 minutes. Some process had been held up but was then released by the feeding stimulus or something related to it. If the enzyme peak were delayed several hours, it could be correlated with the beginning of colchicine treatment. A 3-5 hour delay would relate it to duration of the mitotic cycle (6) or E-cell to B-cell differentiation (22). In short, if a mechanism is postulated in which colchicine delays secretion via mitotic arrest, then this delay should be in terms of hours rather than minutes. A second objection is the unlikelihood of a physiological stimulus, feed-

ing, overcoming colchicine arrest within 30 minutes and then yielding a normal amount of enzyme. Finally, the treated animals had 8 to 11 days to slowly build up a full complement of secretory cells prior to colchicine treatment which means that mitotic arrest would not have affected their number nor would 30 extra minutes in a colchicine-rich environment significantly add to it.

The second alternative can better explain the 30 minute retardation because it places the site(s) of colchicine inhibition more proximal to production of the active protease which is what the delayed peak actually represents. The suggested sites are more logical choices for effecting only a 30 minute delay, also, because the possible mechanisms of action would permit normal secretory cell differentiation in large numbers up to some step within 30 minutes of the "ripe" stage. Colchicine inhibition might then act via depression of protein synthesis or through other of its known actions (8). Finally, the feeding stimulus might then effect release of the inhibition by acting on the functioning of the secretory cell, a system more amenable to rapid, physiological control mechanisms than is the mitotically dividing cell.

Since retardation is present from the start, the initial portions of the two curves are especially important. Enzyme activity in the controls decreases during the first

30 minutes while, in the treated, it increases. This indicates that the feeding stimulus normally causes a removal of active protease from the hepatopancreas within the first half hour but, in the treated specimens, it stimulates a rapid production of the active enzyme which was in a very low concentration in the gland at time 0, prior to feeding.

Protein assays

Protein concentrations possess the same scatter as the protease activities and this is probably due to the same reasons proposed above, i.e. factors related to the molting cycle. Curves of the selected values show rhythmic changes, with some differences from the protease curves, but still provide confirmation of an activity cycle within the hepatopancreas after feeding.

Effects of colchicine In spite of a similar rhythmicity, the effects of colchicine on protein concentration show two important differences from its effects on protease activity. (1) In the original data there is no apparent difference between the two halves of the $6\frac{1}{2}$ hour period and, therefore, the protein concentrations do not reflect the 20% rise observed with protease activity. Moreover, when curves of the selected values are examined, the maxima of control values are nearly always higher than those of the treated, indicating that colchicine reduced the protein con-

centrations in the treated hepatopancreases. (2) In contrast to the protease fluctuations, the protein contents of control and treated animals display a nearly synchronous, in-phase rhythm.

The effects of colchicine on protein concentration are, therefore, opposite to those on protease activity. The drug apparently reduces the amount of protein in the hepatopancreas but it does not alter the phase of the rhythmic changes in protein concentration from that of the controls.

Before analyzing these differences in response to colchicine, the natures of the two parameters must themselves be examined. Protease activity and protein concentration are related to the extent that the enzyme is itself a protein and that both represent an algebraic sum of amounts present after formation and removal; i.e. enzyme activation and enzyme removal, protein synthesis and protein removal. In the hepatopancreas, as in other gland tissues, removal of enzyme and protein are probably cyclic physiological events which agree in rhythm although the amplitudes may differ. Active protease may fluctuate between some maximum and close to zero while the protein would have as its lower limit the mass of structural protein making up the tissue. Differences in the two parameters are much greater in regard to formation. The appearance of new, biuret protein is registered as soon as the enzyme presursors have

reached a stage of synthesis (peptides?) which is measurable by the biuret reaction and will appear as a gradual increase in amount whether the change is in the size of the molecules or their number. It is not restricted to "active protein", it will be increased by synthesis of non-enzymatic protein (e.g. in cell growth), and decreased by degradation of any protein in the system to provide amino acids. Hence, biuret-indicated fluctuations in "new protein" is apt to be influenced by many other changing factors besides active protease molecules, the changes may or may not be cyclic, and the amplitude is expected to be quite low because of the many biuret-positive molecules in the system. Protease activity, conversely, measures only active enzymes and, thus, changes are expected to occur very rapidly as the enzyme molecule passes from the inactive to the active stage. Protease activation is expected to be cyclic as it is the product of a cyclic process, synchronized differentiation of secretory cells. Finally, the amplitude should be great since the concentration of active protease does not have a minimal limit and may even approach zero.

Therefore, although the two parameters are related, and each is composed of a formation and a removal component, biuret-protein concentration is dependent on cyclic removal and, relative to protease activation, a nearly stable synthesis; while casein-protease activity is dependent on

cyclic removal and cyclic activation, and the rhythmicity of each may or may not agree in phase.

Some information about the interaction of the two parameters is obtained by comparing the protease activity curve with the protein concentration curve for each group of animals. For the controls, the two maxima of the protease curve occur simultaneously as the first and third peaks in protein concentration and the minima of the two curves show good correspondence. There is no middle peak in the protease curve corresponding to that of protein but, otherwise, most of the peaks and valleys in the two curves appear to be in phase. In the case of the treated animals, however, the curves are more similar in shape but events in the protein curve precede those in the enzyme curve by about 30 minutes.

The probable reasons for these differences in agreement are as follows: (1) enzyme removal and protein removal are probably the same in all four curves; (2) enzyme activation and protein synthesis may take place independently; thus there need not be agreement between a rising protein concentration and a stationary enzyme activity; (3) enzyme synthesis and enzyme removal may take place simultaneously and thus the resulting curve of enzyme activity may increase, decrease or remain the same depending on the contribution and extent of each factor.

Therefore, in order to determine the true effects of colchicine on protease activity, enzyme activation must be separated from enzyme removal. This was done by calculating specific activities of the protease which largely removed the influence of protein concentration and the results will be discussed shortly.

Tubule contractions Similar rhythms between the protein concentrations in control and treated specimens indicated that colchicine did not retard the cycling although the amount of protein appears to have been decreased. In the above discussion, the protein concentration curves were said to be dependent on two factors but only one of these, protein removal, underwent periodic fluctuations of large magnitude while the other, protein synthesis, was comparatively stable. Thus, the minima of the protein concentration curves are more accurate indicators of the time of protein removal than are the activity curve minima for enzyme removal. Hence the rhythm of the concentration curves is equated to the timing of some gross, physiological event(s) which cause the removal of protein. On the basis of the morphology of the muscle network (described both in the literature and in Part I) and because of statements by Yonge (60) and Ramsay (46) discussed earlier, these rhythmic, physiological changes are identified as tubule contractions. Fluctuations in the protein concentration curves

may therefore be used to follow rhythmic tubule contractions and minimal values of protein concentration indicate times when the collective, contractile state of the hepatopancreas is at its maximum. On this basis, both the control and colchicine-treated glands are maximally contracted for the first time, at 30 minutes, and again, about every $1\frac{1}{2}$ hours for the duration of the $6\frac{1}{2}$ hour period.

Tubule contractions and protease activity Some type of coordination should be expected to exist between enzyme activity and tubule contraction in the normal animal, e.g. production of the enzyme followed by its removal, since most physiological systems follow a logical sequence. In the control animals this coordination is seen in operation. The similarities in rhythm between the protease activity and the protein concentration curves was pointed out above. An explanation for this similarity can now be postulated. The first peak in both curves is at $1\frac{1}{2}$ hours indicating maximum activity and maximum protein at this point in time. This is followed by a sharp drop in protein, identified now as the second, maximum tubule contraction, coincident with a drop in enzyme activity thus indicating the removal of active enzyme from the gland by contraction of the tubules. The middle peak in the protein curve, which has no counterpart in the activity curve, is explained by presence of the next (third) tubule contraction from $3\frac{1}{2}$ to 4 hours while

enzyme formation continues to rise toward its very high value in the second half of the curve. As explained above, if enzyme formation is being carried on at an accelerated rate, even during enzyme removal, the activity curve might not reflect the loss of enzyme. Evidence for only two enzyme activity peaks is obtained from Hirsch and Jacobs' results (22) in which a three hour interval exists, both between the two secretory cell maxima in this region of the curve and between the two associated enzyme activity peaks. In the present study, therefore, one would expect the next activity maximum after the one at $1\frac{1}{2}$ hours to occur at $4\frac{1}{2}$ hours; in this experiment, it occurred at 5 hours which obeys the prediction within reasonable limits. Coincident with this activity peak is a protein peak which drops sharply between 5 and $5\frac{1}{2}$ hours indicating the next (fourth) tubule contraction. This time, the drop is accurately reflected in the protease curve showing that here, in contrast to the previous situation, the cycle of enzyme activation is at its lowest ebb.

In summary, the protease activity curve is composed of two fluctuating parameters whose rhythmicities are superimposed. In the controls, peak enzyme activities occur at $1\frac{1}{2}$ and 5 hours, while maximum, concerted tubule contraction takes place at 30 minutes, 2, 4 and $5\frac{1}{2}$ hours. Since the tubules possess a high concentration of active enzyme at time

0, the superimposition of these two rhythms yields a highly coordinated effort in which active enzyme is formed and removed in proper sequence for efficient and economic transfer of enzymes to the midgut.

Paradox in treated specimens Colchicine treatment does not appear to affect the rhythmicity of tubule contractions; however, the protease activity rhythm is markedly altered from that of the controls. Thus, at 30 minutes and at 2 hours a paradoxical situation is seen in which the activity has maximum values while, from the protein curve, tubule contractions are also at their maximum at the same two points. In the latter half of the curve the situation is less extreme since the opposing maxima are not coincident, but there is still lack of coordination as seen in the controls. Also, this may be an explanation for the "suspicious" double peak in the enzyme curve between $4\frac{1}{2}$ and $5\frac{1}{2}$ hours.

An explanation for this lack of coordination must be found within the first 30 minutes because differences are first noted at time 0. It was postulated above that colchicine interfered with one or more steps in the formation of active enzyme with three likely loci mentioned. If this were true, the treated specimens possessed a very low concentration of active protease at time 0 compared to the controls. Feeding stimulated tubule contractions in both

groups reaching a maximum at 30 minutes. However, because only the controls possessed a large amount of active enzyme, only this group shows a decrease in activity coincident with contraction. Treated specimens, having little activated enzyme to start with, would not reflect the contraction in their activity curve. Moreover, the feeding stimulus in some manner effects completion of the enzyme activation delayed by colchicine. This completion takes 30 minutes and, so, peak activity is seen at 30 minutes. The question now remains, if the tubules were being maximally contracted, why was the newly activated enzyme not removed as it was being formed? One possibility is that the activated enzyme was removed from the tubules but was still present in the ducts and thus included in assays of the entire gland. A second possible answer is that, in the absence of a large mass of active enzyme in the lumen at time 0, the synchronized contraction of the tubules took place on schedule but the force of the contraction was diminished by a lack of intralumenal pressure. In many systems such pressure acts as a stretch-stimulus for greater contraction. If this were true, the amount of material removed from the treated glands during this tubule contractions should be less than in the controls. Examination of the protein concentration curve shows that, in general, there is a lower amplitude of variation in protein in the

treated animals than in the controls, i.e. less protein is being moved out of the gland with each tubule contraction maximum.

In general, therefore, weaker contractions allowed a build-up of active enzyme, thus maintaining lack of coordination between enzyme activation and tubule contraction. The same situation occurs at 2 hours but the two processes tend to approach synchrony toward the end of the period.

Specific enzyme activity

As previously stated, the main advantage in basing activity on protein content is that fluctuations in protein are, to some extent, removed and the picture more closely resembles true enzyme activity. In this study, where fluctuations in both activation and protein concentration occur, the specific activities better represent the variations in enzyme activation separated from changes in protein levels.

Effects of colchicine Except for the first 30 minutes and the extra minimum in the curve for the treated specimens, the two specific activity curves are remarkably similar in that peak enzyme activity occurs at 2 hours and at 4.5 to 5.5 hours.

During the first 30 minutes, enzyme activation decreases in the controls but is increased over threefold in the treated, a difference also suggested in the previous sec-

tions. This difference confirms the previous speculations that (1) colchicine delayed enzyme activation by 30 minutes, (2) this altered the entire protease activity cycle of the treated animals as well as the coordination normally existing between enzyme activation and tubule contraction, and (3) feeding was a stimulus for resumption of the process of enzyme activation which had been retarded at some stage by colchicine. The smooth, "out-of-phase" curve for specific activity confirms both the 30 minute retardation in the treated animals' activity curve and the existence of only two time intervals at which enzyme activation is maximum.

Conclusions

1. Exposure of crayfish to $10^{-7}M$ colchicine for 24 hours effectively blocked cell division in the hepatopancreas tubule.
2. The activity of casein-protease in hepatopancreas homogenates underwent rhythmic fluctuations or cycling after starvation-feeding in the manner described by others.
3. Colchicine did not reduce protease activity even though production of replacement cells was blocked indicating that secretion in the hepatopancreas is not exclusively holocrine.

4. Colchicine retarded rhythmic secretion so that the entire protease activity cycle in treated animals was 30 minutes behind that in the controls.
5. Protein concentration was also cyclic and probably reflects rhythmic tubule contractions. These occur every $1\frac{1}{2}$ hours with the first maximum at 30 minutes after feeding.
6. Fluctuations in enzyme activity reflected two different rhythmic parameters: changes in enzyme activation and tubule contractions. These two are normally coordinated for maximum efficiency in digestive enzyme transport. Colchicine retarded enzyme activity but did not affect tubule contractions thus removing this coordination.
7. Colchicine retardation was probably not related to its mitotic effects but acted on some step(s) in the activation of the casein-protease. After feeding, about 30 minutes were required for the enzyme to complete its activation compared to zero time for the controls' enzyme.

PART III. OBSERVATIONS OF TUBULE CONTRACTIONS

It had been intended, in this section, to study in vitro contractile responses of hepatopancreas tubules to a variety of agents including extracts of nervous tissues, blood from fed animals, various types and degrees of electrical shock, and a variety of drugs with known stimulatory or inhibitory effects in other invertebrate tissues. Thus, the purpose of Part III was to define the physiological responses of the tubules with regard to contraction and to seek relationships between tubule contraction and the cytological and enzymatic changes observed in the previous sections. However, attempts to stimulate or control tubule contractions in vitro were largely unsuccessful. Contraction could not be initiated consistently by any of the methods tried, in spite of the fact that (1) such contractions are always observed in vivo in the glands of fed animals, and (2) isolated tubules often underwent contractions in van Harreveld's solution without obvious external stimulation.

It soon was apparent that several unknowns and variables were involved, the most essential of these being a defined perfusion medium to maintain the tubules in vitro for several hours. Crayfish balanced salt solution was not adequate to prevent cell death and the use of crayfish blood

serum, although capable of maintaining the tubules in vitro for several days, would require sacrificing a large number of animals to obtain sufficient perfusion fluid for each study. Other factors which seemed to be important were the temperature of the system (tubules appeared to degenerate rapidly at room temperature and above) and the incidental, mechanical stimulations produced during dissection and isolation of the tubules.

Because of these problems, experimentation was postponed until a standardized perfusion media and technique could be worked out. In Part III, therefore, in lieu of formal investigations, some general observations of tubule contractions will be presented which were recorded while performing various experiments on the hepatopaneas over a two-year period; and, from these, some conclusions will be drawn."

Appearance during dissection

When the hepatopaneas is removed from a fasted crayfish and its connective tissue covering removed, the individual tubules float free in the perfusion fluid giving the gland a feathery appearance. At this time, the tubules are nearly all in a relaxed state and resemble long, thin fingers extending out from the ducts and having rounded points at their distal ends. The lumen appears as a light streak

running the length of the tubule and the circular muscles provide a regular pattern of faint indentations on the tubules' surfaces.

About one hour after removal, many of the tubules are in a contracted state, but the event occurs too slowly to "see" contractile movements. Along the length of a single tubule various types of constrictions are apparent. Some of these are narrow, as though caused by a single fiber and others are wide, resembling a tight collar. Several constrictions may be closely grouped together or a single constriction may be remote from others. The unconstricted, bulbous portions may be spherical or cylindrical and their length varies considerably. Many times constrictions occur within a few tenths of a millimeter from the blind end but leaving the tip free, thus giving it the appearance of a child's top. The lumen can be observed in the bulbous portions where its diameter increases with that of the tubule.

In vivo appearance after feeding

Several attempts were made to follow materials into the hepatopancreas. The most encouraging of these experiments included feeding the specimen dye-impregnated food (such as liver soaked in the lipid soluble Nile Blue A) and observing movement of the dye in the alimentary tract.

The crayfish is anesthetized in crushed ice for 10 or

15 minutes, its dorsal carapace is removed and the specimen is placed in cold van Harreveld's solution. The hepatopancreas is exposed with a minimum of probing and the individual tubules are observed with a dissecting microscope through the transparent, connective tissue covering of the gland. As the media warms to room temperature, the crayfish begins to recover, its heart rate returns to normal, and blood is gradually replaced by media due to the heart's action.

As the animal recovers, its movements appear normal, in spite of the dorsal opening, and it will accept and ingest food, if starved beforehand. Thus, feeding may take place either before or after exposure of the hepatopancreas tubules. Within a short time after feeding, small globules may be seen entering the main ducts of the hepatopancreas. Fat dyes remain in the globules while water-soluble dyes tend to diffuse into the tissues and cells. After another period of time, tiny droplets of dye are seen to enter the tubules themselves.

The state of tubule contraction is not uniform throughout the gland. Tubules on the dorsal surface of the gland are usually relaxed while those on the inside medial surfaces, especially near the hilum, are observed mainly in the contracted state. Peristalsis or similar waves of contraction were not in evidence in any of the observed glands,

although the rates of movement may have been too slow to follow. In one experiment the animal was fed liver without dye. The dark brown color of the liver was seen first in the ducts and then, progressively, in more distal portions of tubule in the bulbous, non-constricted regions. Although the actual movement was too slow to observe, the dark brown coloration gradually filled the lumen of most of the tubules.

Stimulation experiments

Both electrical and chemical stimulation of the tubules were attempted. For the first, crayfish were dissected to expose the hepatopancreas as described above and electrodes were placed in various regions, including the anterior portion of the stomach where Keim (28) located the ventricular ganglion, various regions of the alimentary tract, and on the gland itself. Repetitive stimuli of 30 volts, when applied to the gland at 5-10 per second for 10 seconds, sometimes resulted in massive contractions of tubules after 30 to 60 minutes, however, the usual result was negative.

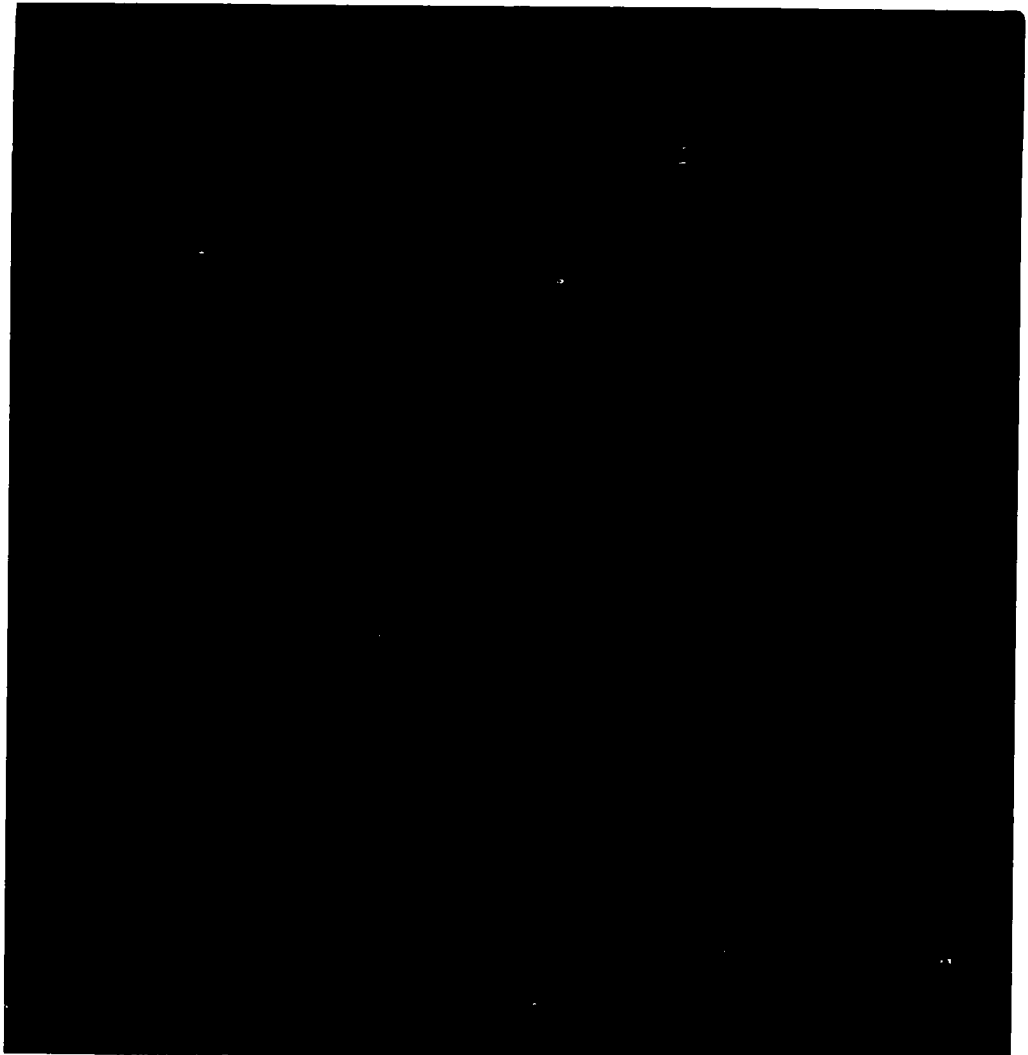
Several drug experiments were attempted in which isolated tubules were exposed to blood from fed or starved animals, nicotine, atropine, ATP, adrenaline, eserine, gamma-amino butyric acid, 5-hydroxytryptamine, and

tubocurarine. All were applied at $10^{-3}M$ strength and all yielded negative results. However, Fig. 44 shows a group of tubules which had been placed in perfusion fluid containing equal concentrations of acetylcholine and 5-hydroxytryptamine. Erspamer (10) reports that this combination initiated rhythmic contractions in several invertebrate muscles which are relaxed by 5-HT alone. Although rhythmic contractions were not observed, this was the only treatment that caused extensive tubule contraction. In addition, while tubules in the other solutions became flaccid and underwent degeneration after a short time, the tubules shown in Fig. 44 had been in the combined drug solution at room temperature for about four hours and showed no signs of degeneration.

Observations during protease studies

In about half the animals used in the casein-protease studies, after removal and preparation of the right gland for homogenization, the left gland was examined for tubule contractions. Notes were taken on the extent of tubule contraction such as "no contraction", "minor contraction near hilum", "50% of tubules in maximum contraction" "extensive contractions over entire gland", etc. Later, these subjective descriptions were assigned a number from an arbitrary scale on which 0 = no contraction, 5 = moderate con-

Fig. 44. Isolated group of tubules in contracted state
After four hours' exposure to 10^{-3} M 5-hydroxy-
tryptamine and acetylcholine at room tempera-
ture, tubules do not appear to be degenerating
as they do in media without these drugs.
(x20)



traction, 10 = maximum contraction. Forty-six observations were recorded with $n = 1$ to 6 animals per time interval. The number of observations and scale average for each interval are as follows:

<u>Time</u>	<u>n</u>	<u>Average contraction</u>
starved	1	5
$\frac{1}{2}$ hr.	2	7.5*
1	3	3.3
$1\frac{1}{2}$	4	5
2	4	8.8*
$2\frac{1}{2}$	4	3.8
3	6	4.2
$3\frac{1}{2}$	4	8.8*
4	3	3.3
$4\frac{1}{2}$	3	8.3*
5	4	6.2
$5\frac{1}{2}$	2	7.5
6	3	10.0*
$6\frac{1}{2}$	3	6.7

As indicated by the asterisks (*), the averages show a maximum at $\frac{1}{2}$, 2, $3\frac{1}{2}$, $4\frac{1}{2}$ and 6 hours; that is, maximal contractions appear at regular intervals of approximately $1\frac{1}{2}$ hours beginning at $\frac{1}{2}$ hour after feeding. The animals are a combination of colchicine-treated and control crayfish.

Conclusions

1. Crayfish perfusion fluid at room temperature is not an adequate system for maintaining isolated hepatopancreas tubules, especially for studies on tubule contraction.

2. Tubule contraction is involved with the movement of food particles into the tubule lumen but the rate of movement is too slow for direct observation.

3. There is some indication that tubule contraction is selectively controlled in different regions of the gland.

4. The tubules are sensitive to electrical and chemical stimulation but the procedures have not been standardized to allow investigations into the mechanism of stimulation or its role in physiological control of the gland.

5. Observations of a limited number of glands after starvation-feeding indicate rhythmic tubule contractions at $1\frac{1}{2}$ hour intervals with the first appearing at $\frac{1}{2}$ hour.

GENERAL DISCUSSION

The original goal of this research was to carry out a series of experiments on a remarkably versatile gland, the crayfish hepatopancreas, which were designed to provide complementary information concerning secretion at many biological levels of organization. When examined realistically, such a goal is more proper to a long-term research plan rather than a dissertation problem. It was therefore modified so that three different areas (general cytology, protease activity and tubule contractions) related by one problem (rhythmic secretion) would be investigated. The wide variety and nature of the experiments and the reduction in their specific objectives require that two questions be asked here: (1) How do the results and conclusions of Parts I, II, and III compare with each other and fit into a general scheme? (2) What are the essential experiments which must be carried out to pursue or enlarge this work and what will be the direction of this future research?

Correlations within the Present Studies

The present investigations have dealt with rhythmic secretion in the hepatopancreas. A general statement of the problem investigated is as follows: Does cyclic secretion of digestive enzymes actually occur in crayfish hepatopancreas as previously described and, if so, what is the basis

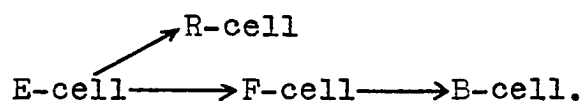
of this rhythmicity?

Answers to this problem, in the literature, have been discussed above. Common to all is the dependence of rhythmicity on the mode secretion and the sequence of cell differentiation in the hepatopancreas tubule. In general, these theories fall into two main categories: (1) Secretion is holocrine and is the final step in the linear differentiation of the secretory cell. Therefore, rhythmic secretion is ultimately dependent on rhythmic mitotic divisions. (2) Secretion is merocrine and the rhythmicity is based on cyclic secretion and restitution of the secretory cell.

Rhythmic secretion in *O. virilis* The rhythmic protease activity after feeding observed in Part II of these studies was nearly identical to that found by Hirsch and Jacobs, proponents of holocrine secretion. The cycling was confirmed by rhythmic protein concentrations in the same tissue samples although specific differences existed between the two activity curves.

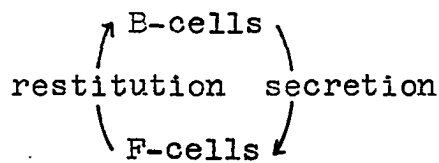
Sequence of cell differentiation The present studies have demonstrated, in Part I, the presence of the same four basic cell types in the tubules of *O. virilis* which were described in other crustaceans, regardless of differences in nomenclature. Evidence was presented, including location in the tubule and morphological similarities between certain of these cell types, which indicated

that the sequence of differentiation postulated by Hirsch and Jacobs was present in O. virilis, namely,



It was also demonstrated that differences between this scheme and those presented by other investigators could be accounted for by important differences in techniques and intervals of time between observations. Thus, up to this point, the results of the present studies indicated holocrine secretion as the operating mechanism.

Mode of secretion Problems arose, however, from the lack of direct evidence for holocrine secretion. In Part I, merocrine secretion was observed in both light and electron microscopic studies of animals fasted for two days while, in fed animals, light microscopy revealed the presence of apocrine secretion. In Part II, colchicine arrest of mitotic divisions, which should have stopped the supply of replacement cells and thus sharply reduced protease activity if secretion was exclusively holocrine, did not have this effect. Thus, Parts I and II indicated a restitutive form of secretion rather than a form resulting in the death of secretory cells. Assuming that only four cell types are present in the tubule and that two of these, E-cells and R-cells are not directly involved in secretion, the following scheme of restitution might occur:



Non-exclusiveness of theories

Thus, the present studies offered evidence for two modes of secretion within the tubule. Arguments can be raised that the original evidence of holocrine secretion was in error and that the presence of linear differentiation, although accurate, merely describes the sequence followed by replacement cells for secretory cells dying from causes other than secretion. This explanation, however, would not account for the sequence of maximum numbers of cell types ending in a peak enzyme activity obtained by these authors and which strongly indicates holocrine secretion.

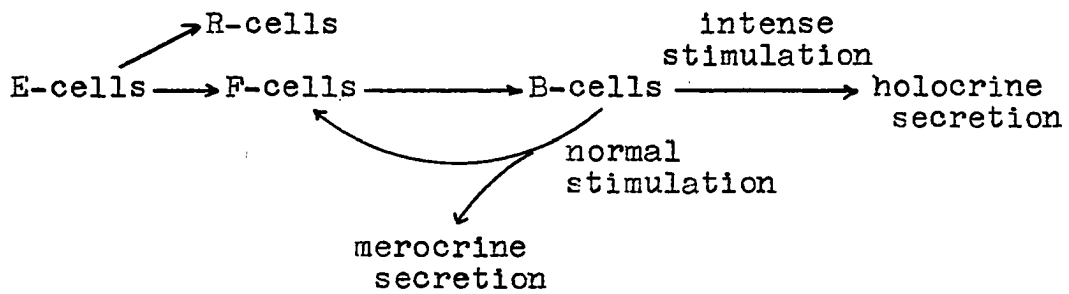
The entire problem rests on the assumed exclusiveness of either type of secretion and this assumption is no longer justified. Kurosumi (32), in redefining the older concepts of modes of secretion in the light of fine structure observations, discusses various examples in which a combination of two or more types of extrusion occur in the same cells. These include the parietal cells of the stomach, thyroid gland cells, mammary gland cells, and cells of the pancreas, among others. Moreover, after intense stimulation, the result is "stressed secretion" and normally merocrine secret-

ing cells will undergo apocrine secretion or even complete loss of their cytoplasmic contents which appears identical to holocrine secretion.

Tubule contractions Does the hepatopancreas tubule possess any mechanism which might result in "stressed secretion"? In Part I, the presence of the muscle network described in the older literature was observed in both light and electron microscopy. Yonge (60) stated that this system was responsible for movements of materials into and out of the tubule. In Part II, evidence was presented for the rhythmic removal of both protease and protein from the tubule. An argument was presented that, of the two, the cyclic protein changes best reflected the timing of this process, that removal was probably due to maximum, concerted contractions by the tubules, and that the latter occurred about every $1\frac{1}{2}$ hours beginning at 30 minutes after feeding. In Part III, simple observations of glands after feeding indicated that tubules actually did follow a rhythmic pattern of maximum contraction and the timing agreed with that indicated by protein removal. Thus, if such contractions were intensely stimulated, as Kurosumi observed, they could result in "stressed secretion" and rupturing or releasing of secretory cells giving the appearance of holocrine secretion. It is suggested that starvation for a long period of time followed by feeding is, indeed, a stressed stimulus.

Vonk (56) criticized the work of Hirsch and Jacobs on this basis, claiming that their results probably did not represent mechanisms in the normal animal.

An integrated scheme A mechanism which would account for both the rhythmicity of secretion and the above observations is now offered. Secretion may be both holocrine and merocrine, the latter term used in the older sense implying restitution of the secretory cell. After moderate stimulation and during short periods between feedings, merocrine secretion is the major mode of extrusion. However, after extreme stimulation, such as starvation-feeding, a large number of secretory cells, packed with secretory material due to lack of stimuli, are ruptured or released by intense contractions of the tubules brought about by feeding, i.e. holocrine secretion. Therefore, secretion is rhythmic because the secretory cells have been synchronized by prolonged starvation followed by massive holocrine secretion and the synchrony is maintained in the waves of replacement cells undergoing differentiation. As the time after feeding increases, the synchrony diminishes, the intensity of tubule contractions returns to normal, and the merocrine mechanisms again become the major mode of secretion. This scheme may be summarized as follows:



Future Work

Even before the above research was carried out, a multitude of interesting experiments were suggested by the unique combination of simple morphology and variety of functions present in the hepatopancreas tubule. Furthermore, the present work indicated several new problems including: from Part I, the effects of eyestalk hormones on the differentiation and fine structure of the fibrillar cell, and fluorescent antibody experiments to localize zymogens in the tubule cells or to compare known substances, such as trypsin inhibitor, with the polysaccharide of the iron granules; from Part II, the effects of colchicine and other mitotic inhibitors on cell migration and on extrusion of secretory materials; and, from Part III, time-lapse cinematography of tubule contractions.

Standard conditions However, the present research revealed that several unknowns were present, both in basic knowledge and technique, which made some desirable approaches untenable. With benefit of hindsight, the neces-

sary preliminary experiments, as well as a long-range plan, can now be outlined which would still utilize the advantages of the system but proceed in a more step-wise fashion.

The first step will be to establish proper conditions for the in vitro maintenance of isolated hepatopancreas tubules for long periods of time. This will be an empirical study possibly involving additions to the crayfish physiological saline solution such as energy sources and buffers, and determining correct concentrations. Another approach is to start with a defined culture media, bring it to the proper osmolality and pH, and then enrich it with crayfish serum. Temperature appears to be critical since isolated tubules usually degenerated quickly at room temperature but were maintained for five days at 14°C until bacterial invasion occurred. Lower temperatures might be obtained by circulating cold perfusion fluid over the specimen on the microscope stage. Finally, proper specimen preparation procedures and dissection techniques will have to be devised to avoid injury or unwanted stimulation of the tubules.

Isolated tubules Once the standard conditions have been defined, the next step would be to incorporate them into a micro-perfusion system for observing isolated tubules with either dissecting or compound microscope. A commercial chamber is now available which holds protozoans or other small specimens between two horizontal coverslips for micro-

scopic observation and through which media may be perfused. A simpler perfusion chamber might also be fashioned from a capillary tube.

This technique would be the key to a wide variety of experiments. (1) The contraction process could be studied by adding drugs to the perfusion fluid and recording the state of contraction at intervals of time or by the use of time-lapse cinematography. Thus, the rate and state of contraction could be placed on a time scale for comparison with other parameters. (2) The perfusion system would facilitate light and electron microscopy studies by permitting almost immediate fixation by perfusing fixatives at the precise moment desired. Thus, contraction, secretion, and cell differentiation could be controlled and studied with far greater precision than previously. (3) If the system were provided with fine, wire leads, some interesting electrophysiological experiments could be carried out. Stimulating electrodes could be used to stimulate the tubules and pick-up electrodes to detect electrical changes during secretion. In either case, the perfusate would be available to analyze for digestive enzymes and other secretory products.

The above techniques have been used with microorganisms and should be readily adaptable to the hepatopancreas. The advantage of the system is that it would allow the simultaneous collection of precise data at many

levels of organization in the hepatopancreas tubule.

Intact animal The results of these experiments on isolated tubules must be confirmed in the intact animal. In the present studies, crayfish were used in late summer when the metabolic and nutritional state of the hepatopancreas was most unstable. Future studies would be carried out in the spring to obtain maximum values of digestive enzyme secretion. Specimens would be maintained at lower temperatures, probably 14°C to 18°C, in running water. Finally, more data would be recorded regarding sex, size and molt state. The same parameters as above would be studied but, in addition to simple stimulation, the role of photoperiods, diurnal cycles and molting might be investigated.

To complete the cytological and physiological data obtained from the above experiments, studies would be carried out on the effects of hormones on tubule cultures. Using such organ cultures, the long-term effects of purified eyestalk extracts on RNA maintenance in the fibrillar cells and the mechanisms involved could be investigated.

The over-all goal of this plan is to correlate data from cytology, physiology and biochemistry in order to present a detailed concept of secretion including mechanisms at all levels of organization in the animal. Secretion is an example of a life process in which all levels, from genes

to muscles, participate and the crayfish hepatopancreas is a system especially suitable for studying life processes at a variety of levels.

SUMMARY AND CONCLUSIONS

Secretion in the crayfish hepatopancreas was investigated in three ways. (1) The morphology and cytochemistry of the hepatopancreas tubule and its epithelium was studied in the living state and using light and electron microscopy. (2) Variations in the production of a trypsin-like protease by this tissue as well as protein concentrations were measured at 30-minute intervals for $6\frac{1}{2}$ hours after feeding and specific enzyme activities were calculated. The effects of colchicine treatment on these parameters were investigated. (3) Observations were made of contractions in individual tubules after various treatments and at intervals after feeding.

The following conclusions were made:

1. The four cell types described by previous investigators are present in *O. virilis*.

2. The fibrillar cell observed with light microscopy owes its appearance to a coarse system of rough endoplasmic reticulum with distended intracisternal spaces which are elongated toward the apical surface in the mature cell.

3. Secretion of digestive enzymes by the hepatopancreas is probably merocrine or apocrine in the normal animal but extensive holocrine secretion occurs immediately after intense stimulation.

4. The secretory cycle includes both linear cell differentiation to replace lost cells and cyclic restitution during merocrine secretion.

5. Rhythmic secretion of digestive enzymes does occur in the hepatopancreas but its source is not entirely due to a secretory cycle within the cells. It is probably a combination of the latter plus rhythmic emptying of the tubules by contractions of the muscle network. In the normal animal, the rhythms of these two processes are coordinated for maximum efficiency of digestive juice transport.

6. Exposure of the intact crayfish to $10^{-7}M$ colchicine for 24 hours arrested mitosis in the distal end of the tubules.

7. Colchicine treatment retarded the entire secretory rhythm by 30 minutes, probably by interfering with some phase of active enzyme formation. It did not affect rhythmic emptying of the tubule.

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ACKNOWLEDGEMENTS

The author is greatly indebted to his major professor, Dr. James R. Redmond, for guidance, support and indulgence of time. Of great influence were Dr. Redmond's contagious fascination with living things and his philosophy that divisions of biology are not exclusive domains, but are special tools which the biologist should select to uncover life's secrets. He did not make the author a specialist; he did make him more curious and reminded him to think broadly.

Completion of this work was favored by fruitful discussions with Doctors John H. D. Bryan and John Arnold of Zoology concerning cytology, cytochemistry, cell differentiation and photomicrography; with Dr. Donald J. Graves of Biochemistry and Biophysics concerning enzyme assay techniques; and with various staff members of the Microscopy Laboratory, Biochemistry and Biophysics concerning electron microscopy. Special thanks in this last group are due Mr. Robert Thomas, who cut some of the E. M. sections for this study.

The author is also grateful to his typist, Mrs. A. J. Terrones, whose sharp eye caught a large number of unusual spellings and other special renditions of English usage.

Finally, the author affectionately thanks and dedicates

this work to his wife, Charlotte Marie, whose patience and encouragement have made it as much her accomplishment as his.